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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C07K 16/28, A61K 39/395, C07K 16/46, C12N 15/13	A1	(11) International Publication Number: WO 00/78815 (43) International Publication Date: 28 December 2000 (28.12.2000)
(21) International Application Number: PCT/US00/17454 (22) International Filing Date: 23 June 2000 (23.06.2000) (30) Priority Data: 09/339,922 24 June 1999 (24.06.1999) US (60) Parent Application or Grant APPLIED MOLECULAR EVOLUTION [/]; (). HUSE, William, D. [/]; (). WU, Herren [/]; (). HUSE, William, D. [/]; (). WU, Herren [/]; (). CADENA, Deborah, L. ; ().		Published
(54) Title: ANTI-'alpha' ₂ 'v'beta' ₂ '3 RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE (54) Titre: ANTICORPS HUMAINS RECOMBINANTS ANTI-ALPHA ₂ VBETA ₂ '3, ACIDES NUCLEIQUES CODANT CES ANTICORPS ET METHODES D'UTILISATION (57) Abstract <p>The invention provides enhanced LM609 grafted antibodies exhibiting selective binding affinity to 'alpha'₂'v'beta'₂'3, or a functional fragment thereof. The invention also provides nucleic acid molecules encoding the enhanced LM609 grafted antibodies. Additionally provided are methods of inhibiting a function of 'alpha'₂'v'beta'₂'3 by contacting 'alpha'₂'v'beta'₂'3 with an enhanced LM609 grafted antibody.</p> (57) Abrégé <p>L'invention concerne des anticorps greffés LM609 améliorés ayant une affinité de liaison sélective pour 'alpha'₂'v'beta'₂'3, ou un fragment fonctionnel desdits anticorps. Elle concerne également des molécules d'acide nucléique codant lesdits anticorps greffés LM609 améliorés, ainsi que des méthodes qu'on met en oeuvre pour inhiber une fonction de 'alpha'₂'v'beta'₂'3 en plaçant 'alpha'₂'v'beta'₂'3 au contact d'un anticorps greffé LM609 amélioré.</p>		

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(19) World Intellectual Property Organization
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(43) International Publication Date
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PCT

(10) International Publication Number
WO 00/78815 A1

(51) International Patent Classification⁷: C07K 16/28, A61K 39/395, C07K 16/46, C12N 15/13

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(21) International Application Number: PCT/US00/17454

(22) International Filing Date: 23 June 2000 (23.06.2000)

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(25) Filing Language: English

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(26) Publication Language: English

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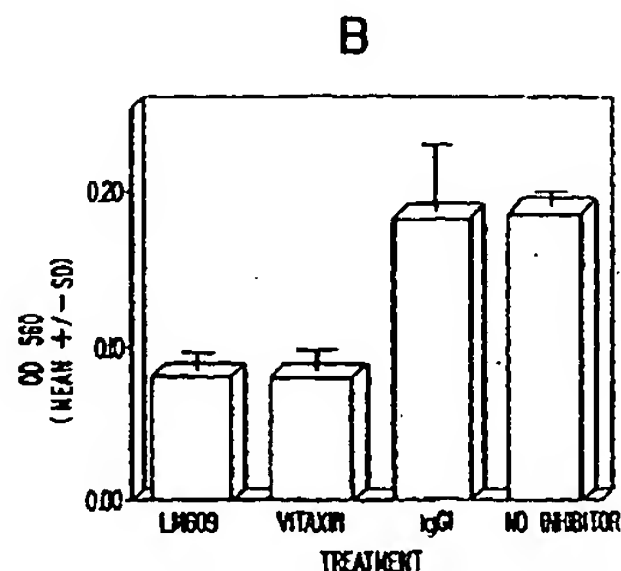
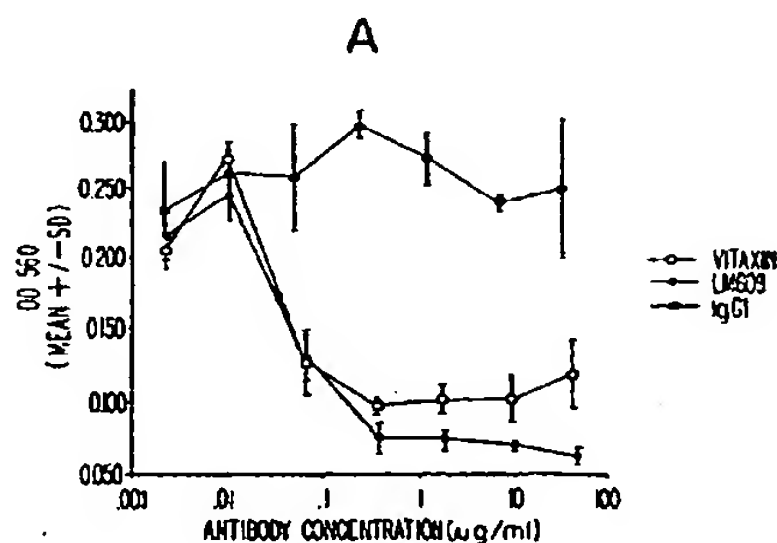
(30) Priority Data:
09/339,922 24 June 1999 (24.06.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/339,922 (CIP)
Filed on 24 June 1999 (24.06.1999)

(81) Designated States (national): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM,

[Continued on next page]

(54) Title: ANTI- $\alpha_3\beta_1$ RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE



(57) Abstract: The invention provides enhanced LM609 grafted antibodies exhibiting selective binding affinity to $\alpha_3\beta_1$, or a functional fragment thereof. The invention also provides nucleic acid molecules encoding the enhanced LM609 grafted antibodies. Additionally provided are methods of inhibiting a function of $\alpha_3\beta_1$ by contacting $\alpha_3\beta_1$ with an enhanced LM609 grafted antibody.

WO 00/78815 A1



HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Description

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ANTI- $\alpha_v\beta_3$, RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS
ENCODING SAME AND METHODS OF USE

BACKGROUND OF THE INVENTION

The present invention relates generally to integrin mediated diseases and, more particularly, to nucleic acids encoding $\alpha_v\beta_3$ -inhibitory monoclonal antibodies and to CDR grafted $\alpha_v\beta_3$ -inhibitory antibodies for the therapeutic treatment of $\alpha_v\beta_3$ -mediated diseases.

Integrins are a class of cell adhesion receptors that mediate both cell-cell and cell-extracellular matrix adhesion events. Integrins consist of heterodimeric polypeptides where a single α chain polypeptide noncovalently associates with a single β chain. There are now about 14 distinct α chain polypeptides and at least about 8 different β chain polypeptides which constitute the integrin family of cell adhesion receptors. In general, different binding specificities and tissue distributions are derived from unique combinations of the α and β chain polypeptides or integrin subunits. The family to which a particular integrin is associated with is usually characterized by the β subunit. However, the ligand binding activity of the integrin is largely influenced by the α subunit. For example, vitronectin binding integrins contain the α_v integrin subunit.

It is now known that the vitronectin binding integrins consist of at least three different α_v containing integrins. These α_v containing integrins include $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$, all of which exhibit different ligand binding specificities. For example, in addition to vitronectin, $\alpha_v\beta_3$ binds to a large variety of

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extracellular matrix proteins including fibronectin, fibrinogen, laminin, thrombospondin, von Willebrand factor, collagen, osteopontin and bone sialoprotein I.

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The integrin $\alpha_v\beta_1$ binds to fibronectin, osteopontin and vitronectin whereas $\alpha_v\beta_3$ is known to bind to vitronectin and osteopontin.

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As cell adhesion receptors, integrins are involved in a variety of physiological processes including, for example, cell attachment, cell migration

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and cell proliferation. Different integrins play

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different roles in each of these biological processes and the inappropriate regulation of their function or activity can lead to various pathological conditions.

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For example, inappropriate endothelial cell proliferation

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during neovascularization of a tumor has been found to be mediated by cells expressing vitronectin binding

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integrins. In this regard, the inhibition of the

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vitronectin-binding integrin $\alpha_v\beta_3$ also inhibits this process of tumor neovascularization. By this same

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criteria, $\alpha_v\beta_3$ has also been shown to mediate the abnormal cell proliferation associated with restenosis and

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granulation tissue development in cutaneous wounds, for example. Additional diseases or pathological states mediated or influenced by $\alpha_v\beta_3$ include, for example,

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metastasis, osteoporosis, age-related macular

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degeneration and diabetic retinopathy, and inflammatory diseases such as rheumatoid arthritis and psoriasis.

Thus, agents which can specifically inhibit vitronectin-binding integrins would be valuable for the therapeutic

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30 treatment of diseases.

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Many integrins mediate their cell adhesive functions by recognizing the tripeptide sequence Arg-Gly-Asp (RGD) found within a large number of extracellular

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5 matrix proteins. A variety of approaches have attempted
to model agents after this sequence to target a
particular integrin-mediated pathology. Such approaches
10 include, for example, the use of RGD-containing peptides
5 and peptide analogues which rely on specificity to be
conferred by the sequences flanking the RGD core
tripeptide sequence. Although there has been some
15 limited success, most RGD-based inhibitors have been
shown to be, at most, selective for the targeted integrin
10 and therefore exhibit some cross-reactivity to other
non-targeted integrins. Such cross-reactive inhibitors
20 therefore lack the specificity required for use as an
efficacious therapeutic. This is particularly true for
previously identified inhibitors of the integrin $\alpha_v\beta_3$.

25 15. Monoclonal antibodies on the other hand exhibit
the specificity required to be used as an effective
therapeutic. Antibodies also have the advantage in that
30 they can be routinely generated against essentially any
desired antigen. Moreover, with the development of
20 combinatorial libraries, antibodies can now be produced
faster and more efficiently than by previously used
35 methods within the art. The use of combinatorial
methodology also allows for the selection of the desired
antibody along with the simultaneous isolation of the
25 encoding heavy and light chain nucleic acids. Thus,
40 further modification can be performed to the
combinatorial antibody without the incorporation of an
additional cloning step.

45 Regardless of the potential advantages
30 associated with the use of monoclonal antibodies as
therapeutics, these molecules nevertheless have the
drawback in that they are almost exclusively derived from
50 non-human mammalian organisms. Therefore, their use as

5 therapeutics is limited by the fact that they will
normally elicit a host immune response. Methods for
substituting the antigen binding site or complementarity
10 determining regions (CDRs) of the non-human antibody into
5 a human framework have been described. Such methods vary
in terms of which amino acid residues should be
substituted as the CDR as well as which framework
15 residues should be changed to maintain binding
specificity. In this regard, it is understood that
10 proper orientation of the β sheet architecture, correct
packing of the heavy and light chain interface and
20 appropriate conformation of the CDRs are all important
for preserving antigen specificity and affinity within
the grafted antibody. However, all of these methods
15 require knowledge of the nucleotide and amino acid
sequence of the non-human antibody and the availability
25 of an appropriately modeled human framework.

30 Thus, there exists a need for the availability
of nucleic acids encoding integrin inhibitory antibodies
20 which can be used as compatible therapeutics in humans.
For $\alpha_v\beta_3$ -mediated diseases, the present invention
satisfies this need and provides related advantages as
35 well.

SUMMARY OF THE INVENTION

40
25 The invention provides enhanced LM609 grafted
antibodies exhibiting selective binding affinity to $\alpha_v\beta_3$,
or a functional fragment thereof. The invention also
45 provides nucleic acid molecules encoding the enhanced
LM609 grafted antibodies. Additionally provided are
30 methods of inhibiting a function of $\alpha_v\beta_3$ by contacting $\alpha_v\beta_3$
with an enhanced LM609 grafted antibody.
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BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 shows the nucleotide and deduced amino acid sequence of the variable region of the antibody Vitaxin. Figure 1A shows the nucleotide and deduced amino acid sequences for the Vitaxin heavy chain variable region (Gln1-Ser117; SEQ ID NOS:1 and 2, respectively) while Figure 1B shows the nucleotide and deduced amino acid sequences for the Vitaxin light chain variable region (Glu1-Lys107; SEQ ID NOS:3 and 4, respectively).

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10 Figure 2 shows the nucleotide and deduced amino acid sequence of the variable region of the monoclonal antibody LM609. Figure 2A shows the nucleotide and deduced amino acid sequence of the LM609 heavy chain variable region (SEQ ID NOS:5 and 6, respectively). The variable region extends from amino acid Glu1 to Ala117. Figure 2B shows the nucleotide and deduced amino acid sequence of the LM609 light chain variable region (SEQ ID NOS:7 and 8, respectively). The variable region of the light chain extends from amino acid Asp1 to Lys107.

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20 Figure 3 shows the competitive inhibition of LM609 IgG binding to the integrin $\alpha_v\beta_3$ with recombinant LM609 Fab. Soluble recombinant murine LM609 Fab fragments were prepared from periplasmic fractions of M13 bacteriophage clones muLM609M13 12 and muLM609M13 29. The periplasm samples were serially diluted, mixed with either 1 ng/ml, 5 ng/ml, or 50 ng/ml of LM609 IgG and then incubated in 96 well plates coated with purified $\alpha_v\beta_3$. Plates were washed and bound LM609 IgG detected with goat anti-murine Fc specific antibody conjugated to alkaline phosphatase. Fab produced by clone muLM609M13 12 inhibits both 1 ng/ml and 5 ng/ml LM609 IgG binding at all concentrations of Fab greater than 1:27 dilution.

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Figure 4 shows the characterization of Vitaxin binding specificity. Figure 4A shows specific binding of Vitaxin to the integrin $\alpha_v\beta_3$, compared to integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_5$. Figure 4B shows the competitive inhibition of LM609 binding to $\alpha_v\beta_3$ by Vitaxin. Figure 4C shows the competitive inhibition of fibrinogen binding to $\alpha_v\beta_3$ by Vitaxin.

Figure 5 shows the inhibition of $\alpha_v\beta_3$ -mediated cell attachment (5A) and migration (5B) by Vitaxin.

Figure 6 shows the reduction in tumor growth due to Vitaxin mediated inhibition of neovascularization. Figure 6A shows the inhibition of the $\alpha_v\beta_3$ -negative Fg and HEP-3 human tumor fragments grown on chick chorioallantoic membranes (CAMs) following Vitaxin treatment. Figure 6B shows the growth inhibition of Vx2 carcinomas implanted subcutaneously in rabbits at two different Vitaxin doses administered 1 day post implantation. Figure 6C similarly shows Vx2 tumor growth inhibition as in Figure 6B, except that four different Vitaxin doses were administered beginning at 7 days post implantation.

Figure 7 shows the nucleotide and deduced amino acid sequence of the light chain variable region of the LM609 grafted antibody fragment (Glu1-Lys107; SEQ ID NOS:31 and 32, respectively). Position 49 of the light chain variable region can at least be either Arg or Met. The nucleotide and deduced amino acid sequence of the heavy chain variable region of the LM609 grafted antibody fragment is shown in Figure 1A (SEQ ID NOS:1 and 2, respectively).

Figure 8 shows the titration of LM609 grafted antibody variants and LM609 grafted Fab on immobilized $\alpha_v\beta_3$. Bacterial cell lysates containing LM609 grafted antibody (closed circles), LM609 grafted antibody variants with improved affinity isolated from the primary libraries (S102, closed squares; Y100, open squares; and Y101, open triangles) or from combinatorial libraries (closed triangles), or an irrelevant Fab (open circles) were titrated on immobilized $\alpha_v\beta_3$.

Figure 9 shows the construction of combinatorial libraries of enhanced LM609 grafted antibody variants containing multiple amino acid substitutions.

Figure 10 shows the inhibition of fibrinogen binding to $\alpha_v\beta_3$ by LM609 grafted antibody variants. Figure 10A shows inhibition of fibrinogen binding to immobilized $\alpha_v\beta_3$. Figure 10B shows correlation of affinity of antibody variants with inhibition of fibrinogen binding.

Figure 11 shows the inhibition of M21 human melanoma cell adhesion to fibrinogen by LM609 grafted antibody variants. Cell binding to 10 $\mu\text{g/ml}$ fibrinogen-coated substrate was assessed in the presence of various concentrations of LM609 grafted Fab (closed triangles) or the enhanced LM609 grafted Fabs S102 (open circles), G102 (closed circles), or C37 (open triangles).

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to nucleic acids encoding the monoclonal antibody (MAb) LM609. This antibody specifically recognizes the integrin $\alpha_v\beta_3$ and

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inhibits its functional activity. The invention is also directed to nucleic acids encoding and to polypeptides comprising non-murine grafted forms of LM609. These grafted antibodies retain the binding specificity and inhibitory activity of the parent murine antibody LM609. The invention is additionally directed to optimized forms of LM609 grafted antibodies that exhibit increased binding affinity and specificity compared to the non-mouse parental forms of the LM609 grafted antibody.

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10 In one embodiment, the hybridoma expressing LM609 was used as a source to generate and clone cDNAs encoding LM609. The heavy and light chain encoding cDNAs were sequenced and their CDR regions were substituted into a human antibody framework to generate the non-murine form of the antibody. The substitution or grafting of the CDRs was performed by codon-based mutagenesis to generate a combinatorial antibody Fab library consisting of members that presented alternative residues at certain positions. Screening of the library resulted in the isolation of Vitaxin. As a grafted antibody containing human framework sequences, it is unlikely that Vitaxin will elicit a host immune response and can therefore be advantageously used for the treatment of $\alpha_v\beta_3$ -mediated diseases.

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25 As used herein, the term "monoclonal antibody LM609" or "LM609" is intended to mean the murine monoclonal antibody specific for the integrin $\alpha_v\beta_3$, which is described by Cheresh, D.A. Proc. Natl. Acad. Sci. USA 84:6471-6475 (1987) and by Cheresh and Spiro J. Biol. Chem. 262:17703-17711 (1987). LM609 was produced against and is reactive with the M21 cell adhesion receptor now known as the integrin $\alpha_v\beta_3$. LM609 inhibits the attachment of M21 cells to $\alpha_v\beta_3$ ligands such as vitronectin,

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5 fibrinogen and von Willebrand factor (Cheresh and Spiro,
supra) and is also an inhibitor of $\alpha_v\beta_3$ -mediated
pathologies such as tumor induced angiogenesis (Brooks et
10 al. Cell 79:1157-1164 (1994), granulation tissue
5 development in cutaneous wound (Clark et al., Am. J.
Pathology, 148:1407-1421 (1996)) and smooth muscle cell
migration such as that occurring during restenosis (Choi
15 et al., J. Vascular Surg., 19:125-134 (1994); Jones et
al., Proc. Natl. Acad. Sci. 93:2482-2487 (1996)).

10 As used herein, the term "Vitaxin" is intended
20 to refer to a non-mouse antibody or functional fragment
thereof having substantially the same heavy and light
chain CDR amino acid sequences as found in LM609. The
25 term "Vitaxin" when used in reference to heavy or light
15 chain polypeptides is intended to refer to a non-mouse
heavy or light chain or functional fragment thereof
having substantially the same heavy or light chain CDR
amino acid sequences as found in the heavy or light chain
30 of LM609, respectively. When used in reference to a
20 functional fragment, not all LM609 CDRs need to be
represented. Rather, only those CDRs that would normally
35 be present in the antibody portion that corresponds to
the functional fragment are intended to be referenced as
the LM609 CDR amino acid sequences in the Vitaxin
25 functional fragment. Similarly, the use of the term
40 "Vitaxin" in reference to an encoding nucleic acid is
intended to refer to a nucleic acid encoding a non-mouse
antibody or functional fragment having substantially the
same nucleotide sequence as the heavy and light chain CDR
45 30 nucleotide sequences and encoding substantially the same
CDR amino acid sequences as found in LM609.

5 As used herein, the term "LM609 grafted
antibody" is intended to refer to a non-mouse antibody or
functional fragment thereof having substantially the same
10 heavy and light chain CDR amino acid sequences as found
5 in LM609 and absent of the substitution of LM609 amino
acid residues outside of the CDRs as defined by Kabat et
al., U.S. Dept. of Health and Human Services, "Sequences
15 of Proteins of Immunological Interest" (1983). The term
"LM609 grafted antibody" or "LM609 grafted" when used in
10 reference to heavy or light chain polypeptides is
intended to refer to a non-mouse heavy or light chain or
20 functional fragment thereof having substantially the same
heavy or light chain CDR amino acid sequences as found in
the heavy or light chain of LM609, respectively, and also
15 absent of the substitution of LM609 residues outside of
the CDRs as defined by Kabat et al., *supra*. When used in
25 reference to a functional fragment, not all LM609 CDRs
need to be represented. Rather, only those CDRs that
would normally be present in the antibody portion that
30 corresponds to the functional fragment are intended to be
referenced as the LM609 CDR amino acid sequences in the
LM609 grafted functional fragment. Similarly, the term
35 "LM609 grafted antibody" or "LM609 grafted" used in
reference to an encoding nucleic acid is intended to
25 refer to a nucleic acid encoding a non-mouse antibody or
functional fragment being absent of the substitution of
40 LM609 amino acids outside of the CDRs as defined by Kabat
et al., *supra* and having substantially the same
nucleotide sequence as the heavy and light chain CDR
30 nucleotide sequences and encoding substantially the same
45 CDR amino acid sequences as found in LM609 and as defined
by Kabat et al., *supra*.

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The term "grafted antibody" or "grafted" when used in reference to heavy or light chain polypeptides or functional fragments thereof is intended to refer to a heavy or light chain or functional fragment thereof having substantially the same heavy or light chain CDR of a donor antibody, respectively, and also absent of the substitution of donor amino acid residues outside of the CDRs as defined by Kabat et al., *supra*. When used in reference to a functional fragment, not all donor CDRs need to be represented. Rather, only those CDRs that would normally be present in the antibody portion that corresponds to the functional fragment are intended to be referenced as the donor CDR amino acid sequences in the functional fragment. Similarly, the term "grafted antibody" or "grafted" when used in reference to an encoding nucleic acid is intended to refer to a nucleic acid encoding an antibody or functional fragment, being absent of the substitution of donor amino acids outside of the CDRs as defined by Kabat et al., *supra* and having substantially the same nucleotide sequence as the heavy and light chain CDR nucleotide sequences and encoding substantially the same CDR amino acid sequences as found in the donor antibody and as defined by Kabat et al., *supra*.

The meaning of the above terms are intended to include minor variations and modifications of the antibody so long as its function remains uncompromised. Functional fragments such as Fab, F(ab)₂, Fv, single chain Fv (scFv) and the like are similarly included within the definition of the terms LM609 and Vitaxin. Such functional fragments are well known to those skilled in the art. Accordingly, the use of these terms in describing functional fragments of LM609 or the Vitaxin antibody are intended to correspond to the definitions

5 well known to those skilled in the art. Such terms are
described in, for example, Harlow and Lane, Antibodies: A
10 Laboratory Manual, Cold Spring Harbor Laboratory, New
York (1989); Molec. Biology and Biotechnology: A
5 Comprehensive Desk Reference (Myers, R.A. (ed.), New
York: VCH Publisher, Inc.); Huston et al., Cell
Biophysics, 22:189-224 (1993); Plückthun and Skerra,
15 Meth. Enzymol., 178:497-515 (1989) and in Day, E.D.,
Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc.,
10 New York, NY (1990).

20 As with the above terms used for describing
functional fragments of LM609, Vitaxin and a LM609
grafted antibody, the use of terms which reference other
LM609, Vitaxin or LM609 grafted antibody domains,
25 functional fragments, regions, nucleotide and amino acid
15 sequences and polypeptides or peptides, is similarly
intended to fall within the scope of the meaning of each
term as it is known and used within the art. Such terms
30 include, for example, "heavy chain polypeptide" or "heavy
20 chain", "light chain polypeptide" or "light chain",
"heavy chain variable region" (V_H) and "light chain
variable region" (V_L) as well as the term "complementarity
35 determining region" (CDR).

In the case where there are two or more
40 25 definitions of a term which is used and/or accepted
within the art, the definition of the term as used herein
is intended to include all such meanings unless
explicitly stated to the contrary. A specific example is
45 the use of the term "CDR" to describe the non-contiguous
30 antigen combining sites found within the variable region
of both heavy and light chain polypeptides. This
particular region has been described by Kabat et al.,
50 *supra*, and by Chothia et al., J. Mol. Biol. 196:901-917

(1987) and by MacCallum et al., J. Mol. Biol. 262:732-745 (1996) where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of LM609, Vitaxin, LM609 grafted antibodies or variants thereof is intended to be within the scope of the term as defined and used herein. The amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison.

Table 1: CDR Definitions

	<u>Kabat</u> ¹	<u>Chothia</u> ²	<u>MacCallum</u> ³
V _H CDR1	31-35	26-32	30-35
V _H CDR2	50-65	53-55	47-58
V _H CDR3	95-102	96-101	93-101
V _L CDR1	24-34	26-32	30-36
V _L CDR2	50-56	50-52	46-55
V _L CDR3	89-97	91-96	89-96

¹ Residue numbering follows the nomenclature of Kabat et al., *supra*

² Residue numbering follows the nomenclature of Chothia et al., *supra*

³ Residue numbering follows the nomenclature of MacCallum et al., *supra*

As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to a reference sequence. Such considerable degree, amount or extent of sequence

5 identity is further considered to be significant and
meaningful and therefore exhibit characteristics which
are definitively recognizable or known. Thus, a
10 nucleotide sequence which is substantially the same
5 nucleotide sequence as a heavy or light chain of LM609,
Vitaxin, or a LM609 grafted antibody including fragments
thereof, refers to a sequence which exhibits
15 characteristics that are definitively known or
recognizable as encoding or as being the amino acid
10 sequence of LM609, Vitaxin or a LM609 grafted antibody.
Minor modifications thereof are included so long as they
20 are recognizable as a LM609, Vitaxin or a LM609 grafted
antibody sequence. Similarly, an amino acid sequence
which is substantially the same amino acid sequence as a
15 heavy or light chain of Vitaxin, a LM609 grafted antibody
or functional fragment thereof, refers to a sequence
which exhibits characteristics that are definitively
known or recognizable as representing the amino acid
30 sequence of Vitaxin or a LM609 grafted antibody and minor
20 modifications thereof:

When determining whether a nucleotide or amino
35 acid sequence is substantially the same as Vitaxin or a
LM609 grafted antibody, consideration is given to the
number of changes relative to the Vitaxin or LM609
25 grafted antibody together with whether the function is
40 maintained. For example, a single amino acid change in a
3 amino acid CDR or several changes in a 16 amino acid
CDR are considered to be substantially the same if $\alpha\beta_1$
binding function is maintained. Thus, a nucleotide or
45 amino acid sequence is substantially the same if it
30 exhibits characteristics that are definitively known or
recognizable as representing the nucleotide or amino acid
sequence of Vitaxin or a LM609 grafted antibody and minor
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modifications thereof as long as Vitaxin or LM609 grafted antibody function is maintained.

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As used herein, the term "fragment" when used in reference to a nucleic acid encoding LM609, Vitaxin or a LM609 grafted antibody is intended to mean a nucleic acid having substantially the same sequence as a portion of a nucleic acid encoding LM609, Vitaxin or a LM609 grafted antibody. The nucleic acid fragment is sufficient in length and sequence to selectively hybridize to an LM609, a Vitaxin or a LM609 grafted antibody encoding nucleic acid or a nucleotide sequence that is complementary to an LM609, Vitaxin or LM609 grafted antibody encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for nucleic acid blot or solution hybridization. The meaning of the term is also intended to include regions of nucleotide sequences that do not directly encode LM609 polypeptides such as the introns, and the untranslated region sequences of the LM609 encoding gene.

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As used herein, the term "functional fragment" when used in reference to Vitaxin, to a LM609 grafted antibody or to heavy or light chain polypeptides thereof is intended to refer to a portion of Vitaxin or a LM609 grafted antibody including heavy or light chain polypeptides which still retains some or all of the $\alpha_v\beta_3$ binding activity, $\alpha_v\beta_3$ binding specificity and/or integrin $\alpha_v\beta_3$ -inhibitory activity. Such functional fragments can include, for example, antibody functional fragments such as Fab, F(ab)₂, Fv, single chain Fv (scFv). Other functional fragments can include, for example, heavy or light chain polypeptides, variable region polypeptides or CDR polypeptides or portions thereof so long as such

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functional fragments retain binding activity, specificity or inhibitory activity. The term is also intended to include polypeptides encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics so long as such polypeptides retain functional activity as defined above.

As used herein, the term "enhanced" when used in reference to Vitaxin, a LM609 grafted antibody or a functional fragment thereof is intended to mean that a functional characteristic of the antibody has been altered or augmented compared to a reference antibody so that the antibody exhibits a desirable property or activity. An antibody exhibiting enhanced activity can exhibit, for example, higher affinity or lower affinity binding, or increased or decreased association or dissociation rates compared to a reference antibody. An antibody exhibiting enhanced activity can also exhibit increased stability such as increased half-life in a particular organism. For example, an antibody activity can be enhanced to increase stability by decreasing susceptibility to proteolysis. If enhanced activity such as higher affinity binding or increased stability is desired, mutations can be introduced into framework or CDR amino acid residues and the resulting antibody variants screened for higher affinity binding to $\alpha_v\beta_3$ or increased stability relative to a reference antibody such as the LM609 grafted parent antibody. An antibody exhibiting enhanced activity can also exhibit lower affinity binding, including decreased association rates or increased dissociation rates, if desired. An enhanced antibody exhibiting lower affinity binding is useful, for example, for penetrating a solid tumor. In contrast to a higher affinity antibody, which would bind to the

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peripheral regions of the tumor but would be unable to penetrate to the inner regions of the tumor due to its high affinity, a lower affinity antibody would be advantageous for penetrating the inner regions of the tumor.

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The invention provides a nucleic acid encoding a heavy chain polypeptide for Vitaxin or a functional fragment thereof. Also provided is a nucleic acid encoding a light chain polypeptide for Vitaxin or a functional fragment thereof. The nucleic acids consist of substantially the same heavy or light chain variable region nucleotide sequences as those shown in Figure 1A and 1B (SEQ ID NOS:1 and 3, respectively) or a fragment thereof.

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Vitaxin, including functional fragments thereof, is a non-mouse antibody which exhibits substantially the same binding activity, binding specificity and inhibitory activity as LM609. The Vitaxin Fv Fragment was produced by functionally replacing CDRs within human heavy and light chain variable region polypeptides with the CDRs derived from LM609. Functional replacement of the CDRs was performed by recombinant methods known to those skilled in the art. Such methods are commonly referred to as CDR grafting and are the subject matter of U.S. Patent No. 5,225,539. Such methods can also be found described in "Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications in Man," Clark, M. (ed.), Nottingham, England: Academic Titles (1993).

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Briefly, LM609 nucleic acid fragments having substantially the same nucleotide and encoding substantially the same amino acid sequence of each of the

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heavy and light chain CDRs were synthesized and substituted into each of the respective human chain encoding nucleic acids. To maintain functionality of the newly derived Vitaxin antibody, modifications were performed within the non-CDR framework region. These individual changes were made by generating a population of CDR grafted heavy and light chain variable regions wherein all possible changes outside of the CDRs were represented and then selecting the appropriate antibody by screening the population for binding activity. This screen resulted in the selection of the Vitaxin antibody described herein.

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The nucleotide sequences of the Vitaxin heavy and light chain variable regions are shown in Figures 1A and 1B, respectively. These sequences correspond substantially to those that encode the heavy and light chain variable region polypeptides of Vitaxin. These Vitaxin nucleic acids are intended to include both the sense and anti-sense strands of the Vitaxin encoding sequences. Single- and double-stranded nucleic acids are similarly included as well as non-coding portions of the nucleic acid such as introns, 5'- and 3'-untranslated regions and regulatory sequences of the gene for example.

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As shown in Figure 1A, the Vitaxin heavy chain variable region polypeptide is encoded by a nucleic acid of about 351 nucleotides in length which begins at the amino terminal Gln1 residue of the variable region through to Ser117. This Vitaxin heavy chain variable region encoding nucleic acid is joined to a human IgG1 constant region to yield a coding region of 1431 nucleotides which encodes a heavy chain polypeptide of 477 total amino acids. Shown in Figure 1B is the Vitaxin light chain variable region polypeptide which is encoded

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5 by a nucleic acid of about 321 nucleotides in length
beginning at the amino terminal Glu1 residue of the
variable region through to Lys107. This Vitaxin light
10 chain variable region nucleic acid is joined to a human
5 kappa construct region to yield a coding region of 642
nucleotides which code for a light chain polypeptide of
214 total amino acids.

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Minor modification of these nucleotide
sequences are intended to be included as heavy and light
10 chain Vitaxin encoding nucleic acids and their functional
20 fragments. Such minor modifications include, for
example, those which do not change the encoded amino acid
sequence due to the degeneracy of the genetic code as
well as those which result in only a conservative
25 substitution of the encoded amino acid sequence.
Conservative substitutions of encoded amino acids
include, for example, amino acids which belong within the
following groups: (1) non-polar amino acids (Gly, Ala,
30 Val, Leu, and Ile); (2) polar neutral amino acids (Cys,
20 Met, Ser, Thr, Asn, and Gln); (3) polar acidic amino
acids (Asp and Glu); (4) polar basic amino acids (Lys,
Arg and His); and (5) aromatic amino acids (Phe, Trp,
35 Tyr, and His). Other minor modifications are included
within the nucleic acids encoding Vitaxin heavy and light
25 chain polypeptides so long as the nucleic acid or encoded
40 polypeptides retain some or all of their function as
described herein.

45 Thus, the invention also provides a nucleic
acid encoding a Vitaxin heavy chain or functional
30 fragment thereof wherein the nucleic acid encodes
substantially the same heavy chain variable region amino
acid sequence of Vitaxin as that shown in Figure 1A (SEQ
50 ID NO:2) or a fragment thereof. Similarly, the invention

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also provides a nucleic acid encoding a Vitaxin light chain or functional fragment thereof wherein the nucleic acid encodes substantially the same light chain variable region amino acid sequence of Vitaxin as that shown in Figure 1B (SEQ ID NO:4) or a fragment thereof.

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In addition to conservative substitutions of amino acids, minor modifications of the Vitaxin encoding nucleotide sequences which allow for the functional replacement of amino acids are also intended to be included within the definition of the term. The substitution of functionally equivalent amino acids encoded by the Vitaxin nucleotide sequences is routine and can be accomplished by methods known to those skilled in the art. Briefly, the substitution of functionally equivalent amino acids can be made by identifying the amino acids which are desired to be changed, incorporating the changes into the encoding nucleic acid and then determining the function of the recombinantly expressed and modified Vitaxin polypeptide or polypeptides. Rapid methods for making and screening multiple simultaneous changes are well known within the art and can be used to produce a library of encoding nucleic acids which contain all possible or all desired changes and then expressing and screening the library for Vitaxin polypeptides which retain function. Such methods include, for example, codon based mutagenesis, random oligonucleotide synthesis and partially degenerate oligonucleotide synthesis.

Codon based mutagenesis is the subject matter of U.S. Patent Nos. 5,264,563 and 5,523,388 and is advantageous for the above procedures since it allows for the production of essentially any and all desired frequencies of encoded amino acid residues at any and all

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particular codon positions within an oligonucleotide. Such desired frequencies include, for example, the truly random incorporation of all twenty amino acids or a specified subset thereof as well as the incorporation of a predetermined bias of one or more particular amino acids so as to incorporate a higher or lower frequency of the biased residues compared to other incorporated amino acid residues. Random oligonucleotide synthesis and partially degenerate oligonucleotide synthesis can similarly be used for producing and screening for functionally equivalent amino acid changes. However, due to the degeneracy of the genetic code, such methods will incorporate redundancies at a desired amino acid position. Random oligonucleotide synthesis is the coupling of all four nucleotides at each nucleotide position within a codon whereas partially degenerate oligonucleotide synthesis is the coupling of equal portions of all four nucleotides at the first two nucleotide positions, for example, and equal portions of two nucleotides at the third position. Both of these latter synthesis methods can be found described in, for example, Cwirla et al., Proc. Natl. Acad. Sci. USA 87:6378-6382, (1990) and Devlin et al., Science 249:404-406, (1990).

Identification of amino acids to be changed can be accomplished by those skilled in the art using current information available regarding the structure and function of antibodies as well as available and current information encompassing methods for CDR grafting procedures. For example, CDRs can be identified within the donor antibody by any or all of the criteria specified in Kabat et al., *supra*, Chothia et al., *supra*, and/or MacCallum et al., *supra*, and any or all non-identical amino acid residues falling outside of

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these CDR sequences can be changed to functionally equivalent amino acids. Using the above described methods known within the art, any or all of the non-identical amino acids can be changed either alone or in combination with amino acids at different positions to incorporate the desired number of amino acid substitutions at each of the desired positions. The Vitaxin polypeptides containing the desired substituted amino acids are then produced and screened for retention or augmentation of function compared to the unsubstituted Vitaxin polypeptides. Production of the substituted Vitaxin polypeptides can be accomplished by, for example, recombinant expression using methods known to those skilled in the art. Those Vitaxin polypeptides which exhibit retention or augmentation of function compared to unsubstituted Vitaxin are considered to contain minor modifications of the encoding nucleotide sequence which result in the functional replacement of one or more amino acids.

The functional replacement of amino acids is beneficial when producing grafted antibodies having human framework sequences since it allows for the rapid identification of equivalent amino acid residues without the need for structural information or the laborious procedures necessary to assess and identify which amino acid residues should be considered for substitution in order to successfully transfer binding function from the donor. Moreover, it eliminates the actual step-wise procedures to change and test the amino acids identified for substitution. Essentially, using the functional replacement approach described above, all non-identical amino acid residues between the donor and the human framework can be identified and substituted with any or all other possible amino acid residues at each

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non-identical position to produce a population of substituted polypeptides containing all possible or all desired permutations and combinations. The population of substituted polypeptides can then be screened for those substituted polypeptides which retain function. Using the codon based mutagenesis procedures described above, the generation of a library of substituted amino acid residues and the screening of functionally replaced residues has been used for the rapid production of grafted therapeutic antibodies as well as for the rapid alteration of antibody affinity. Such procedures are exemplified in, for example, Rosok et al., J. Biol. Chem. 271:22611-22618 (1996) and in Glaser et al., J. Immunol. 149:3903-3913 (1992), respectively.

15 In addition to framework residues, amino acids in one or more CDRs can be functionally replaced to allow identification of a modified LM609 grafted antibody having enhanced activity. Using the methods described above for framework residues, amino acid substitutions can similarly be introduced into one or more CDRs in an LM609 grafted antibody. The modified LM609 grafted antibody can be tested for binding activity to determine whether $\alpha\beta$ binding activity is maintained. The modified LM609 grafted antibody can be further tested to determine if activity has been enhanced. Functional replacement of amino acid residues in one or more CDRs therefore allows the identification of an enhanced LM609 grafted antibody having a desirable property such as enhanced activity.

30 To generate modified LM609 grafted antibodies and select those with enhanced activity, several approaches can be employed in the selection of the number of residues within a CDR to mutate as well as the number of CDRs within a LM609 grafted antibody to modify. The

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choice of selection criteria for mutagenesis of CDRs will depend on the need and desired application of the enhanced antibody. For example, one or a few amino acid positions within a single CDR can be modified to contain selected amino acids at that position. Alternatively, the targeted amino acid positions can be modified to contain all possible amino acids at that position. The resultant population of modified antibodies can then be screened for enhanced activity.

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10 The construction of modified LM609 grafted antibody populations can also be made where all amino acids positions within a CDR have been mutated to contain all possible amino acids and where amino acid positions within multiple CDRs have been modified to contain variant amino acid residues. In this way, populations can be constructed which range from 2 to $>10^7$ unique members. The larger the population, the more efficient will be the selection of an enhanced LM609 grafted antibody since there will be a larger number of different antibodies within the population. However, a small population of modified LM609 grafted antibodies can be made and successfully used for the selection of enhanced LM609 grafted antibodies. The size of the population of modified LM609 grafted antibodies will depend on the need of a particular application and can be determined by one skilled in the art.

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 The generation of modified LM609 grafted antibodies can be achieved by introducing amino acid substitutions into one or more CDRs of an LM609 grafted antibody. For example, single amino acid substitutions can be systematically introduced into a CDR by changing a given amino acid in the CDR to any or all amino acids. Amino acid substitutions can also be introduced into all

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amino acid positions in one or more of the CDRs or in all of the CDRs, generating a population of modified LM609 grafted antibody variants. This population of modified LM609 grafted antibody variants having single amino acid substitutions can be screened to identify those variants that maintain $\alpha_v\beta_3$ binding activity. The variants having $\alpha_v\beta_3$ binding activity can be further characterized to identify those variants having enhanced activity. Such a systematic approach to introducing single amino acid substitutions and generating a population of LM609 grafted antibody variants to screen for enhanced LM609 grafted antibodies having high affinity binding to $\alpha_v\beta_3$ is described in Example VI.

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In addition to generating a population of modified LM609 grafted antibody variants, a particular CDR or a particular amino acid in a CDR can be selected to introduce one or more amino acid substitutions. For example, sequence homology or a structural model can be used to identify particular amino acid positions to introduce amino acid substitutions. In this example, only one or a few modified LM609 grafted antibody variants are generated and screened for binding activity to $\alpha_v\beta_3$. One of skill in the art will know or can determine whether it is desirable to generate a large population of modified LM609 grafted antibody variants or to generate a limited number of modified LM609 grafted antibody variants to screen and identify an enhanced LM609 grafted antibody having enhanced activity.

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In addition to identifying enhanced LM609 grafted antibodies by generating a population of modified LM609 grafted antibodies having single amino acid substitutions in a CDR and screening for enhanced activity, enhanced LM609 grafted antibody variants can

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5 also be generated by combining two or more mutations,
each known to independently result in enhanced activity,
into a single antibody. When there are more than two
10 mutations, an efficient way to identify combinations of
5 mutations which further augment activity is to construct
all possible combinations and permutations and then
select for those with enhanced activity. For example,
15 two single mutations in one or more CDRs can be combined
to generate a new modified LM609 grafted antibody having
10 two CDR mutations and screened to determine if the $\alpha_v\beta_2$
binding activity is increased over that of the single
20 mutants. Similarly, three mutations can be combined and
the resulting modified LM609 grafted antibody screened
for enhanced binding activity. Using such an approach of
15 combining CDR mutations, a new population of modified
LM609 grafted antibody variants can be generated by
25 incorporating all combinations of the single CDR
mutations resulting in enhanced activity into new
modified LM609 grafted antibody variants and screening to
30 obtain an optimized enhanced LM609 grafted antibody.
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35 An iterative, step-wise approach to identifying
an enhanced LM609 grafted antibody is advantageous in
that it allows the identification of an antibody having
optimal binding activity without the need to generate and
25 screen a large number of modified LM609 grafted antibody
variants. For example, using the approach described in
40 Examples VI and VII in which single mutants were
identified and combined into a new population of LM609
grafted antibody variants, enhanced LM609 grafted
45 antibodies having higher affinity were identified by
30 generating 2592 unique variants. In contrast, complete
randomization of a single eight amino acid residue CDR
would require $>10^{10}$ unique variants. Therefore, such an
50 iterative approach allows identification of enhanced
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LM609 grafted antibodies having enhanced activity such as high affinity binding by generating a relatively small number of unique modified LM609 grafted antibody variants and screening and identifying those enhanced LM609 grafted antibody variants exhibiting high affinity binding.

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An iterative, step-wise approach to identifying enhanced LM609 variants can also be performed using additional steps. Instead of generating all combinations of single amino acid mutations, the single amino acid mutations can be combined in pairs to generate all combinations of double mutants and screened for activity. Those double mutants having enhanced activity can be combined with any or all single mutants to generate triple mutants that are screened for enhanced binding activity. Each iterative round of generating modified LM609 grafted antibody variants can incorporate additional single mutations, and the resulting modified LM609 grafted antibodies can be screened for enhanced activity. The step-wise generation of LM609 grafted antibody variants can thus be used to identify an optimized LM609 grafted antibody. Additionally, such an iterative approach also allows for the identification of numerous enhanced antibodies which exhibit a range of different, enhanced binding activities.

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An optimized LM609 grafted antibody can also be referred to as an LM609-like grafted antibody or an $\alpha_v\beta_3$ -specific grafted antibody and is recognizable because the antibody or functional fragments thereof retains the functional characteristics of LM609. For example, enhanced LM609 grafted antibody variants, which have a single amino acid substitution and have enhanced activity, can be identified and correlated with a

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specific amino acid substitution. These amino acid substitutions can be combined to generate a new modified LM609 grafted antibody that is tested for activity. Such a combination of advantageous CDR amino acid substitutions can result in an optimized LM609 grafted antibody with multiple CDRs having at least one amino acid substitution or a single CDR having multiple amino acid substitutions, where the modified LM609 grafted antibody has enhanced activity.

Enhanced LM609 grafted antibodies, particularly those optimized by functional replacement of amino acid residues in the CDRs, have desirable enhanced properties such as increased affinity. For example, an optimized LM609 grafted antibody having increased affinity will have higher affinity than the parent antibody used for introducing functional replacement of amino acids. Higher affinity is determined relative to a reference antibody having a similar structure. For example, if the optimized LM609 grafted antibody is an intact antibody containing two heavy chains and two light chains, then higher affinity is determined relative to the intact parent LM609 grafted antibody. Similarly, if the optimized LM609 grafted antibody is an Fab, then higher affinity is determined relative to the Fab of the parent LM609 grafted antibody.

Although it is not necessary to proceed through multiple optimization steps to obtain a high affinity LM609 grafted antibody, in general, the increase in affinity can correlate with the number of modifications within and between CDRs as well as with the number of optimization steps. Therefore, LM609 grafted antibodies will exhibit a variety of ranges. For example, LM609 grafted antibodies having enhanced affinity will have up

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to about 2-fold higher affinity or greater, generally greater than about 2- to 5-fold higher affinity such as greater than about 4- to 5-fold higher affinity or about 5- to 10-fold higher affinity than the reference antibody. Particularly, a LM609 grafted antibody having enhanced affinity will have greater than about 10- to 50-fold higher affinity, greater than about 50-fold higher affinity, or greater than about 100-fold higher affinity than the reference antibody.

As described above, functional replacement of CDR amino acid residues can be used to identify LM609 grafted antibodies exhibiting higher affinity than a parent LM609 grafted antibody. Methods discussed above or below for introducing minor modifications into Vitaxin or LM609 grafted antibody encoding nucleotide sequences can similarly be used to generate a library of modified LM609 grafted antibody variants, including methods such as codon based mutagenesis, random oligonucleotide synthesis and partially degenerate oligonucleotide synthesis. For example, codon based mutagenesis has been used to generate such a library of modified LM609 grafted antibody variants having single amino acid substitutions (see Example VI).

After generating a library of modified LM609 grafted antibody variants, the variants can be expressed and screened for binding activity to $\alpha_v\beta_3$. Methods well known to those skilled in the art related to determining antibody-antigen interactions are used to screen for modified LM609 grafted antibodies exhibiting binding activity to $\alpha_v\beta_3$ (Harlow and Lane, *supra*). For example, an ELISA method has been used to screen a library of modified LM609 grafted antibody variants to identify those variants that maintained $\alpha_v\beta_3$ binding activity (see

5 Example VI). Only those modified LM609 grafted
antibodies that maintain $\alpha_v\beta_3$ binding activity are
considered for further characterization.

10 Modified LM609 grafted antibodies having $\alpha_v\beta_3$
5 binding activity can be further characterized to
determine which modified LM609 grafted antibody has
enhanced activity. The type of assay used to assess
15 enhanced activity depends on the particular desired
characteristic. For example, if altered binding activity
10 is desired, then binding assays that allow determination
of binding affinity are used. Such assays include
20 binding assays, competition binding assays and surface
plasmon resonance as described in Example VI.

25 Introduction of single amino acid substitutions
15 into CDRs of LM609 grafted antibodies can be used to
generate a library of modified LM609 grafted antibodies
and screen for binding activity to $\alpha_v\beta_3$. Those modified
30 LM609 grafted antibodies exhibiting binding activity to
 $\alpha_v\beta_3$ can then be further characterized to identify
20 enhanced LM609 grafted antibodies exhibiting enhanced
activity such as higher binding affinity. For example,
35 using such an approach, a number of enhanced LM609
grafted antibodies having single amino acid substitutions
were generated using the heavy chain variable region
40 shown in Figure 1a (SEQ ID NO:2) and the light chain
variable region shown in Figure 7 (SEQ ID NO:32), and
LM609 grafted antibodies were identified displaying 2 to
45 13-fold improved affinity over the parent LM609 grafted
antibody (see Example VI).

30 Following identification of enhanced LM609
grafted antibodies having a single amino acid
50 substitution, the amino acid mutations can be combined to

5 further enhance activity. Methods discussed above for
introducing single amino acid substitutions into CDRs can
similarly be applied to combine amino acid substitutions.
10 For example, a combinatorial library of amino acid
5 mutations that resulted in enhanced $\alpha\beta$ binding affinity
was generated using degenerate oligonucleotides and two
site hybridization mutagenesis as described in Example
15 VII. Enhanced LM609 grafted antibodies containing
multiple CDR amino acid substitutions were generated
10 using the heavy chain variable region shown in Figure 1a
(SEQ ID NO:2) and the light chain variable region shown
20 in Figure 7 (SEQ ID NO:32), and LM609 grafted antibodies
were identified having 20-fold higher affinity to greater
than 90-fold higher affinity than the parent LM609
15 grafted antibody.

25 In addition to combining CDR amino acid
substitutions to generate an enhanced or optimized LM609
grafted antibody, CDR amino acid substitutions can also
30 be combined with framework mutations that contribute
20 desirable properties to a LM609 grafted antibody. Thus,
mutations in CDR or framework regions that enhance
35 activity can be combined to further optimize LM609
grafted antibodies.

40 The invention further provides fragments of
25 Vitaxin heavy and light chain encoding nucleic acids
wherein such fragments consist substantially of the same
nucleotide or amino acid sequence as the variable region
of Vitaxin heavy or light chain polypeptides. The
45 variable region of the Vitaxin heavy chain polypeptide
30 consists essentially of nucleotides 1-351 and of amino
acid residues Gln1 to Ser117 of Figure 1A (SEQ ID NOS:1
and 2, respectively). The variable region of the Vitaxin
50 light chain polypeptide consists essentially of

5 nucleotides 1-321 and of amino acid residues Glu1 to
Lys107 of Figure 1B (SEQ ID NOS:3 and 4, respectively).
The termini of such variable region encoding nucleic
10 acids is not critical so long as the intended purpose and
5 function remains the same.

Fragments additional to the variable region
15 nucleic acid fragments are provided as well. Such
fragments include, for example, nucleic acids consisting
substantially of the same nucleotide sequence as a CDR of
10 a Vitaxin heavy or light chain polypeptide. Sequences
corresponding to the Vitaxin CDRs include, for example,
20 those regions defined by Kabat et al., *supra*, and/or
those regions defined by Chothia et al., *supra*, as well
as those defined by MacCallum et al., *supra*. The Vitaxin
25 CDR fragments for each of the above definitions
correspond to the nucleotides set forth below in Table 2.
The nucleotide sequence numbering is taken from the
30 primary sequence shown in Figures 1A and 1B (SEQ ID NOS:1
and 3) and conforms to the definitions previously set
20 forth in Table 1.

35 Table 2: Vitaxin CDR Nucleotide Residues

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V _H CDR1	91-105	76-96	88-105
40 V _H CDR2	148-198	157-168	139-177
25 V _H CDR3	295-318	298-315	289-315
V _L CDR1	70-102	76-96	88-108
V _L CDR2	148-168	148-156	136-165
45 V _L CDR3	265-291	271-288	265-288

Similarly, the Vitaxin CDR fragments for each
30 of the above definitions correspond to the amino acid
50 residues set forth below in Table 3. The amino acid

residue number is taken from the primary sequence shown in Figures 1A and 1B (SEQ ID NOS:2 and 4) and conforms to the definitions previously set forth in Table 1.

Table 3: Vitaxin CDR Amino Acid Residues

		<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
5				
15	V _H CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
	V _H CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
	V _H CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
	V _L CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
20	10 V _L CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
	V _L CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Thus, the invention also provides nucleic acid fragments encoding substantially the same amino acid sequence as a CDR of a Vitaxin heavy or light chain polypeptide.

Nucleic acids encoding Vitaxin heavy and light chain polypeptides and fragments thereof are useful for a variety of diagnostic and therapeutic purposes. For example, the Vitaxin nucleic acids can be used to produce Vitaxin antibodies and functional fragments thereof having binding specificity and inhibitory activity against the integrin $\alpha_v\beta_3$. The antibody and functional fragments thereof can be used for the diagnosis or therapeutic treatment of $\alpha_v\beta_3$ -mediated disease. Vitaxin and functional fragments thereof can be used, for example, to inhibit binding activity or other functional activities of $\alpha_v\beta_3$ that are necessary for progression of an $\alpha_v\beta_3$ -mediated disease. Other functional activities necessary for progression of $\alpha_v\beta_3$ -mediated disease include, for example, the activation of $\alpha_v\beta_3$, $\alpha_v\beta_3$ -mediated signal transduction and the $\alpha_v\beta_3$ -mediated prevention of

5 apoptosis. Advantageously, however, Vitaxin comprises
non-mouse framework amino acid sequences and as such is
less antigenic in regard to the induction of a host
10 immune response. The Vitaxin nucleic acids of the
5 inventions can also be used to model functional
equivalents of the encoded heavy and light chain
polypeptides.

15 Thus, the invention provides Vitaxin heavy
chain and Vitaxin light chain polypeptides or functional
10 fragments thereof. The Vitaxin heavy chain polypeptide
20 exhibits substantially the same amino acid sequence as
that shown in Figure 1A (SEQ ID NO:2) or functional
fragment thereof whereas the Vitaxin light chain
25 polypeptide exhibits substantially the same amino acid
15 sequence as that shown in Figure 1B (SEQ ID NO:4) or
functional fragment thereof. Also provided is a Vitaxin
antibody or functional fragment thereof. The antibody is
30 generated from the above heavy and light chain
polypeptides or functional fragments thereof and exhibits
20 selective binding affinity to $\alpha_v\beta_3$.

35 The invention provides a nucleic acid encoding
a heavy chain polypeptide for a LM609 grafted antibody.
Also provided is a nucleic acid encoding a light chain
polypeptide for a LM609 grafted antibody. The nucleic
40 25 acids consist of substantially the same heavy chain
variable region nucleotide sequence as that shown in
Figure 1A (SEQ ID NO:1) and substantially the same light
chain variable region nucleotide sequence as that shown
45 in Figure 7 (SEQ ID NO:31) or a fragment thereof.

30 LM609 grafted antibodies, including functional
fragments thereof, are non-mouse antibodies which exhibit
50 substantially the same binding activity, binding

5 specificity and inhibitory activity as LM609. The LM609
grafted antibody Fv fragments described herein are
produced by functionally replacing the CDRs as defined by
10 Kabat et al. , hereinafter referred to as "Kabat CDRs,"
5 within human heavy and light chain variable region
polypeptides with the Kabat CDRs derived from LM609.
Functional replacement of the Kabat CDRs is performed by
15 the CDR grafting methods previously described and which
is the subject matter of U.S. Patent No. 5,225,539,
10 *supra*. Substitution of amino acid residues outside of
the Kabat CDRs can additionally be performed to maintain
20 or augment beneficial binding properties so long as such
amino acid substitutions do not correspond to a donor
amino acid at that particular position. Such
15 substitutions allow for the modulation of binding
properties without imparting any mouse sequence
characteristics onto the antibody outside of the Kabat
CDRs. Although the production of such antibodies is
described herein with reference to LM609 grafted
30 antibodies, the substitution of such non-donor amino
acids outside of the Kabat CDRs can be utilized for the
production of essentially any grafted antibody. The
35 production of LM609 grafted antibodies is described
further below in Example V.

25 The nucleotide sequences of the LM609 grafted
antibody heavy and light chain variable regions are shown
40 in Figures 1A and 7, respectively. These sequences
correspond substantially to those that encode the heavy
and light chain variable region polypeptides of a LM609
45 grafted antibody. These nucleic acids are intended to
include both the sense and anti-sense strands of the
LM609 grafted antibody encoding sequences. Single- and
50 double-stranded nucleic acids are similarly included as
well as non-coding portions of the nucleic acid such as

introns, 5'- and 3'-untranslated regions and regulatory sequences of the gene for example.

The nucleotide and amino acid residue boundaries for a LM609 grafted antibody are identical to those previously described for Vitaxin. For example, a LM609 grafted antibody heavy chain variable region polypeptide is encoded by a nucleic acid of about 351 nucleotides in length which begins at the amino terminal Gln1 residue of the variable region through to Ser117 (Figure 1A, SEQ ID NOS:1 and 2, respectively). The LM609 grafted antibody light chain variable region polypeptide is encoded by a nucleic acid of about 321 nucleotides in length beginning at the amino terminal Glu1 residue of the variable region through to Lys107 (Figure 7, SEQ ID NOS:31 and 32, respectively). As with Vitaxin, minor modification of these nucleotide sequences are intended to be included as heavy and light chain variable region encoding nucleic acids and their functional fragments.

Thus, the invention also provides a nucleic acid encoding a LM609 grafted antibody heavy chain wherein the nucleic acid encodes substantially the same heavy chain variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) or fragment thereof. Similarly, the invention also provides a nucleic acid encoding a LM609 grafted antibody light chain wherein the nucleic acid encodes substantially the same light chain variable region amino acid sequence as that shown in Figure 7 (SEQ ID NO:32) or fragment thereof.

In addition to conservative substitutions of amino acids, minor modifications of the LM609 grafted antibody encoding nucleotide sequences which allow for the functional replacement of amino acids are also

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intended to be included within the definition of the term. Identification of amino acids to be changed can be accomplished by those skilled in the art using current information available regarding the structure and function of antibodies as well as available and current information encompassing methods for CDR grafting procedures. The substitution of functionally equivalent amino acids encoded by the LM609 grafted antibody nucleotide sequences is routine and can be accomplished by methods known to those skilled in the art. As described previously, such methods include, for example, codon based mutagenesis, random oligonucleotide synthesis and partially degenerate oligonucleotide synthesis and are beneficial when producing grafted antibodies since they allow for the rapid identification of equivalent amino acid residues without the need for structural information.

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The invention further provides fragments of LM609 grafted antibody heavy and light chain encoding nucleic acids wherein such fragments consist substantially of the same nucleotide or amino acid sequence as the variable region of a LM609 grafted antibody heavy or light chain polypeptide. As with Vitaxin, the termini of such variable region encoding nucleic acids is not critical so long as the intended purpose and function remains the same.

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Fragments additional to the variable region nucleic acid fragments are provided as well and include, for example, nucleic acids consisting substantially of the same nucleotide sequence as a CDR of a LM609 grafted antibody heavy or light chain polypeptide. As with Vitaxin, sequences corresponding to the LM609 grafted antibody CDRs include, for example, those regions defined

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5 by Kabat et al., *supra*, Chothia et al., *supra*, as well as
those defined by MacCallum et al., *supra*. The LM609
grafted antibody CDR regions will be similar to those
10 described previously for Vitaxin. Moreover, such regions
5 are well known and can be determined by those skilled in
the art given the LM609 sequences and teachings provided
herein. Thus, the invention also provides nucleic acid
15 fragments encoding substantially the same amino acid
sequence as a CDR of a LM609 grafted antibody heavy or
10 light chain polypeptide.

20 As with Vitaxin, nucleic acids encoding LM609
grafted antibody heavy and light chain polypeptides and
fragments thereof are useful for a variety of diagnostic
and therapeutic purposes. For example, LM609 grafted
25 antibody encoding nucleic acids can be used to produce
15 recombinant antibodies and functional fragments thereof
having binding specificity and inhibitory activity
against the integrin $\alpha_v\beta_3$. The antibody and functional
30 fragments thereof can be used for the diagnosis or
20 therapeutic treatment of $\alpha_v\beta_3$ -mediated disease. Such
diseases and methods of use for anti- $\alpha_v\beta_3$ antibodies have
35 been described previously in reference to Vitaxin and are
equally applicable to the LM609 grafted antibodies
described herein.

40 25 Thus, the invention provides LM609 grafted
antibody heavy chain and Vitaxin light chain polypeptides
or functional fragments thereof. The LM609 grafted
antibody heavy chain polypeptide exhibits substantially
45 the same amino acid sequence as that shown in Figure 1A
30 (SEQ ID NO:2) or functional fragment thereof whereas the
LM609 grafted antibody light chain polypeptide exhibits
substantially the same amino acid sequence as that shown
50 in Figure 7 (SEQ ID NO:32). Also provided is a LM609

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grafted antibody or functional fragment thereof. The antibody is generated from the above heavy and light chain polypeptides or functional fragments thereof and exhibits selective binding affinity to $\alpha_v\beta_3$.

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5 The invention provides an enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$. The enhanced LM609 grafted antibody contains at least one amino acid substitution in one or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of the enhanced LM609 grafted antibody is maintained or enhanced.

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To identify enhanced LM609 grafted antibodies, a library of modified LM609 grafted antibodies was generated as described above and in Example VI. Initially, LM609 CDRs were identified and selected to introduce single amino acid substitutions. Utilizing the numbering system of Kabat et al., *supra*, the CDR residues selected for mutagenesis were V_H CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34) (Gly²⁶-Ser³⁵); V_H CDR2 Trp-Val-Ala-Lys-Val-Ser-Ser-Gly-Gly-Gly (SEQ ID NO:36) and Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID NO:38) (Trp⁴⁷-Gly⁶⁵); V_H CDR3 Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Tyr (SEQ ID NO:40) (Ala⁹³-Tyr¹⁰²); V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-His-Leu-His-Trp-Tyr (SEQ ID NO:42) (Gln²⁴-Tyr³⁶); V_L CDR2 Leu-Leu-Ile-Arg-Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:44) (Leu⁴⁶-Ser⁵⁶); and V_L CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-His-Thr (SEQ ID NO:46) (Gln⁸⁹-Thr⁹⁷).

5 The nucleotide sequences encoding the CDR
residues selected for mutagenesis were V_H CDR1 GGA TTC ACC
TTC AGT AGC TAT GAC ATG TCT (SEQ ID NO:33); V_H CDR2 TGG
10 GTC GCA AAA GTT AGT AGT GGT GGT GGT (SEQ ID NO:35) and
5 AGC ACC TAC TAT TTA GAC ACT GTG CAG GGC (SEQ ID NO:37); V_H
CDR3 GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC (SEQ ID
NO:39); V_L CDR1 CAG GCC AGC CAA AGT ATT AGC AAC CAC CTA
15 CAC TGG TAT (SEQ ID NO:41); V_L CDR2 CTT CTC ATC CGT TAT
CGT TCC CAG TCC ATC TCT (SEQ ID NO:43); and V_L CDR3 CAA
10 CAG AGT GGC AGC TGG CCT CAC ACG (SEQ ID NO:45).

20 Single amino acid substitutions can be
introduced into the CDRs of an LM609 grafted antibody to
generate a population of modified LM609 grafted
antibodies. For example, every amino acid in one or more
25 CDRs can be mutated to any or all amino acids to generate
a population of modified LM609 grafted antibodies and the
population screened for $\alpha_v\beta_3$ binding activity. Although
30 this population is generated by mutating amino acids in
CDRs, populations can also be constructed where changes
20 are made in the framework region residues or in both the
CDRs and the framework. Such mutations in the variable
35 regions can be made separately, in combination, or step-
wise. Thus, the invention also provides for an enhanced
LM609 grafted antibody, where the amino acid substitution
25 is in the CDR or in the framework region.

40 The invention additionally provides an enhanced
LM609 grafted antibody exhibiting enhanced binding
affinity. Enhanced LM609 grafted antibodies exhibiting
45 enhanced binding affinity include those containing at
30 least one of the following CDRs having single amino acid
substitutions:

50 a V_H CDR1 selected from the group consisting of Gly-Thr-
Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:48), Gly-Phe-

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Thr-Trp-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:50) and Gly-Phe-Thr-Phe-Leu-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:52);

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a V_H CDR2 selected from the group consisting of Trp-Val-Ala-Lys-Val-Lys-Ser-Gly-Gly-Gly (SEQ ID NO:54), Ser-Thr-

5 Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ ID NO:56) and Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Glu-Gly (SEQ ID NO:58);

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a V_H CDR3 selected from the group consisting of Ala-Arg-His-Asn-His-Gly-Ser-Phe-Ala-Tyr (SEQ ID NO:60), Ala-Arg-His-Asn-Tyr-Gly-Ser-Tyr-Ala-Tyr (SEQ ID NO:62), Ala-Arg-

10 His-Asn-Tyr-Gly-Ser-Phe-Asp-Tyr (SEQ ID NO:64), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Tyr-Tyr (SEQ ID NO:66), Ala-Arg-

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His-Asn-Tyr-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:68), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Thr (SEQ ID NO:70), Ala-Arg-

His-Asn-Tyr-Gly-Ser-Phe-Ala-Asp (SEQ ID NO:72), Ala-Arg-

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15 His-Asn-Tyr-Gly-Ser-Phe-Ala-Glu (SEQ ID NO:74), Ala-Arg-

His-Asn-Tyr-Gly-Ser-Phe-Ala-Met (SEQ ID NO:76), Ala-Arg-

His-Asn-Tyr-Gly-Ser-Phe-Ala-Gly (SEQ ID NO:78) and Ala-

Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Ala (SEQ ID NO:80);

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the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His-

20 Trp-Tyr (SEQ ID NO:82); the V_L CDR2 Leu-Leu-Ile-Arg-Tyr-

Ser-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:84); and

a V_L CDR3 selected from the group consisting of Gln-Gln-

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Ser-Asn-Ser-Trp-Pro-His-Thr (SEQ ID NO:86), Gln-Gln-Ser-

Thr-Ser-Trp-Pro-His-Thr (SEQ ID NO:88), Gln-Gln-Ser-Gly-

25 Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90) and Gln-Gln-Ser-Gly-

Ser-Trp-Pro-Gln-Thr (SEQ ID NO:92).

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The nucleotide sequences encoding the CDRs

having single amino acid substitutions were V_H CDR1 GGA

ACT ACC TTC AGT AGC TAT GAC ATG TCT (SEQ ID NO:47), GGA

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30 TTC ACC TGG AGT AGC TAT GAC ATG TCT (SEQ ID NO:49), and

GGA TTC ACC TTC CTG AGC TAT GAC ATG TCT (SEQ ID NO:51); V_H

CDR2 TGG GTC GCA AAA GTT AAA AGT GGT GGT GGT (SEQ ID

NO:53), AGC ACC TAC TAT CCT GAC ACT GTG CAG GGC (SEQ ID

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NO:55), and AGC ACC TAC TAT TTA GAC ACT GTG GAG GGC (SEQ

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ID NO:57); V_H CDR3 GCA AGA CAT AAC CAT GGC AGT TTT GCT TAC
(SEQ ID NO:59), GCA AGA CAT AAC TAC GGC AGT TAT GCT TAC
(SEQ ID NO:61), GCA AGA CAT AAC TAC GGC AGT TTT GAT TAC
(SEQ ID NO:63), GCA AGA CAT AAC TAC GGC AGT TTT TAT TAC
5 (SEQ ID NO:65), GCA AGA CAT AAC TAC GGC AGT TTT GCT TCT
(SEQ ID NO:67), GCA AGA CAT AAC TAC GGC AGT TTT GCT ACT
(SEQ ID NO:69), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAT
(SEQ ID NO:71), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAG
(SEQ ID NO:73), GCA AGA CAT AAC TAC GGC AGT TTT GCT ATG
10 (SEQ ID NO:75), GCA AGA CAT AAC TAC GGC AGT TTT GCT GGG
(SEQ ID NO:77), and GCA AGA CAT AAC TAC GGC AGT TTT GCT
GCT (SEQ ID NO:79); V_L CDR1 CAG GCC AGC CAA AGT ATT AGC
AAC TTT CTA CAC TGG TAT (SEQ ID NO:81); V_L CDR2 CTT CTC
ATC CGT TAT TCT TCC CAG TCC ATC TCT (SEQ ID NO:83); and V_L
15 CDR3 CAA CAG AGT AAT AGC TGG CCT CAC ACG (SEQ ID NO:85),
CAA CAG AGT ACT AGC TGG CCT CAC ACG (SEQ ID NO:87), CAA
CAG AGT GGC AGC TGG CCT CTG ACG (SEQ ID NO:89) and CAA
CAG AGT GGC AGC TGG CCT CAG ACG (SEQ ID NO:91).

Enhanced LM609 grafted antibodies having CDRs
20 with single amino acid substitutions and higher affinity
binding than the parent LM609 grafted antibody can also
be identified, where the corresponding amino acid
35 mutations are combined to generate new modified LM609
grafted antibodies. Identification is performed by
25 screening for $\alpha_v\beta_3$ binding activity. In some
combinations, the LM609 grafted antibody will comprise at
least one CDR having two or more amino acid
substitutions. The invention provides an enhanced LM609
grafted antibody containing at least one of the following
45 30 CDRs containing multiple amino acid substitutions: a V_H
CDR3 selected from the group consisting of Ala-Arg-His-
Asn-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:94); Ala-Arg-His-
Asn-His-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:96); Ala-Arg-His-

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Asn-Tyr-Gly-Ser-Phe-Tyr-Glu (SEQ ID NO:98); and Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:100).

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The nucleotide sequences encoding the CDRS having multiple amino acid substitutions were V_H CDR3 GCA
5 AGA CAT AAC CAT GGC AGT TTT GCT TCT (SEQ ID NO:93), GCA
AGA CAT AAC CAT GGC AGT TTT TAT TCT (SEQ ID NO:95), GCA
15 AGA CAT AAC TAC GGC AGT TTT TAT GAG (SEQ ID NO:97), and
GCA AGA CAT AAC TAC GGC AGT TTT TAT TCT (SEQ ID NO:99).

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The invention also provides an enhanced LM609
10 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, wherein the enhanced LM609 grafted antibody contains at least one amino acid substitution in two or more CDRs of a LM609 grafted heavy chain variable region
25 polypeptide or a LM609 grafted light chain variable
15 region polypeptide.

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An enhanced LM609 grafted antibody containing at least one amino acid substitution in two or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable

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20 region polypeptide can include an LM609 grafted antibody containing the combination of CDRs selected from the group consisting of: the V_L CDR1 SEQ ID NO:57 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ
40 ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:52; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:51; the V_L CDR1 SEQ ID NO:57 and
45 the V_H CDR3 SEQ ID NO:52; the V_L CDR3 SEQ ID NO:59, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L
30 CDR3 SEQ ID NO:61 and V_H CDR3 SEQ ID NO:50; and the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and V_H CDR3
50 SEQ ID NO:50.

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In addition to enhanced LM609 grafted antibodies containing two or more CDRs having single amino acid substitutions, the invention also provides enhanced LM609 grafted antibodies wherein at least one of the CDRs has two or more amino acid substitutions.

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Enhanced LM609 grafted antibodies having at least one CDR with two or more amino acid substitutions can include those containing the combination of CDRs selected from the group consisting of: the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:64; the V_L CDR3 SEQ ID NO:61 and the V_H CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61 and the V_H CDR3 SEQ ID NO:65; and the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_L CDR3 SEQ ID NO:66.

The invention additionally provides a high affinity LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$. The high affinity LM609 grafted antibody contains at least one amino acid substitution in one or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of the high affinity LM609 grafted antibody is enhanced.

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High affinity antibodies can include those containing the combination of CDRs selected from the group consisting of: the V_L CDR1 SEQ ID NO:57 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID

5 NO:52; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44
and the V_H CDR3 SEQ ID NO:51; the V_L CDR1 SEQ ID NO:57 and
the V_H CDR3 SEQ ID NO:52; the V_L CDR3 SEQ ID NO:59, the V_H
10 CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L
5 CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H
CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61 and V_H CDR3
SEQ ID NO:50; the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID
15 NO:44 and V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57,
the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:63; the
10 V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H
CDR3 SEQ ID NO:64; the V_L CDR3 SEQ ID NO:61 and the V_H
20 CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61 and the V_H
CDR3 SEQ ID NO:65; and the V_L CDR3 SEQ ID NO:61, the V_H
CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:66.

25 15 As described above, enhanced LM609 antibodies
can be further modified by introducing additional
mutations in one or more CDRs or framework residues. As
disclosed herein, the enhanced LM609 grafted antibody
30 clone 6H6 (Table 10) was further modified by introducing
20 mutations into one or more CDRs (see Example VIII).
These 6H6 variants were found to have high affinity
binding to $\alpha_v\beta_3$ and were resistant to proteolysis.

40 25 The invention further provides enhanced LM609
grafted antibodies comprising the V_H CDR2 Lys-Val-Ser-Ser-
Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ
ID NO:104); the V_H CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser
(SEQ ID NO:106); or the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-
Ser-Asn-Phe-Leu-His (SEQ ID NO:110). The invention also
45 provides a nucleic acid molecule comprising a nucleotide
30 sequence selected from the group consisting of SEQ ID
NO:103, SEQ ID NO:105, and SEQ ID NO:109.

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The invention also provides an enhanced LM609 grafted antibody comprising the V_H CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V_H CDR2 Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID NO:102); the V_H CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:106); the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-His-Leu-His (SEQ ID NO:108); the V_L CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the V_L CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90).

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10 The invention also provides a nucleic acid molecule comprising the nucleotide sequence referenced as SEQ ID NO:33 encoding a V_H CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the nucleotide sequence referenced as SEQ ID NO:107 encoding a V_L CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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20 The invention additionally provides an enhanced LM609 grafted antibody comprising the V_H CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V_H CDR2 Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID NO:102); the V_H CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:106); the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His (SEQ ID NO:110); the V_L CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the V_L CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90).

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30 The invention additionally provides a nucleic acid molecule comprising the nucleotide sequence referenced as SEQ ID NO:33 encoding a V_H CDR1; the

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nucleotide sequence referenced as SEQ ID NO:101 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the nucleotide sequence referenced as SEQ ID NO:109 encoding a V_L CDR1; the

5 nucleotide sequence referenced as SEQ ID NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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The invention further provides an enhanced LM609 grafted antibody comprising the V_H CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V_H CDR2 Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ ID NO:104); the V_H CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:106); the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His (SEQ ID NO:110); the V_L

15 CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the V_L CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90).

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The invention further provides a nucleic acid molecule comprising the nucleotide sequence referenced as

20 SEQ ID NO:33 encoding a V_H CDR1; the nucleotide sequence referenced as SEQ ID NO:103 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the nucleotide sequence referenced as SEQ ID NO:109 encoding a V_L CDR1; the nucleotide sequence

25 referenced as SEQ ID NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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The invention additionally provides a nucleic acid encoding an enhanced LM609 grafted antibody

30 exhibiting selective binding affinity to $\alpha_v\beta_3$. The enhanced LM609 grafted antibody encoded by the nucleic acid contains at least one amino acid substitution in one

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5 or more CDRs of a LM609 grafted heavy chain variable
region polypeptide or a LM609 grafted light chain
variable region polypeptide, wherein the $\alpha_v\beta_3$ binding
10 affinity of the enhanced LM609 grafted antibody is
5 maintained or enhanced.

15 The invention further provides a nucleic acid
encoding a high affinity LM609 grafted antibody
exhibiting selective binding affinity to $\alpha_v\beta_3$. The high
affinity LM609 grafted antibody encoded by the nucleic
10 acid contains at least one amino acid substitution in one
20 or more CDRs of a LM609 grafted heavy chain variable
region polypeptide or a LM609 grafted light chain
variable region polypeptide, wherein the $\alpha_v\beta_3$ binding
affinity of the high affinity LM609 grafted antibody is
25 enhanced.
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The invention provides a nucleic acid encoding
30 a heavy chain polypeptide for monoclonal antibody LM609
or functional fragment thereof. Also provided is a
nucleic acid encoding a light chain polypeptide for
20 monoclonal antibody LM609 or a functional fragment
35 thereof. The nucleic acids consist of substantially the
same heavy or light chain variable region nucleotide
sequences as that shown in Figure 2A and 2B (SEQ ID NOS:5
and 7, respectively) or a fragment thereof.

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25 As described previously, monoclonal antibody
LM609 has been shown in the art to have binding activity
to the integrin $\alpha_v\beta_3$. Although specificity can in
45 principle be generated towards essentially any target,
LM609 is an integrin inhibitory antibody that exhibits
30 substantial specificity and inhibitory activity to a
single member within an integrin family. In this case,
50 LM609 exhibits substantial specificity and inhibitory

5 activity to the $\alpha_v\beta_3$ integrin within the β_3 family. The
amino acid or nucleotide sequence of monoclonal antibody
10 LM609 has never been previously isolated and
characterized.

5 The isolation and characterization of LM609
encoding nucleic acids was performed by techniques known
15 to those skilled in the art and which are described
further below in the Examples. Briefly, cDNA from
hybridoma LM609 was generated and used as the source for
10 which to isolate LM609 encoding nucleic acids. Isolation
was performed by first determining the N-terminal amino
20 acid sequence for each of the heavy and light chain
polypeptides and then amplifying by PCR the antibody
encoding sequences from the cDNA. The 5' primers were
25 reverse translated to correspond to the newly determined
N-terminal amino acid sequences whereas the 3' primers
corresponded to sequences substantially similar to
30 antibody constant region sequences. Amplification and
cloning of the products resulted in the isolation of the
20 nucleic acids encoding heavy and light chains of LM609.

35 The nucleotide sequences of the LM609 heavy and
light chain variable region sequences are shown in Figure
2A and 2B, respectively. These sequences correspond
substantially to those that encode the variable region
40 25 heavy and light chain polypeptides of LM609. As with the
Vitaxin nucleic acids, these LM609 nucleic acids are
intended to include both sense and anti-sense strands of
the LM609 encoding sequences. Single- and double-
45 stranded nucleic acids are also include as well as
30 non-coding portions of the nucleic acid such as introns,
5'- and 3'-untranslated regions and regulatory sequences
of the gene for example.

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As shown in Figure 2A, the LM609 heavy chain variable region polypeptide is encoded by a nucleic acid of about 351 nucleotides in length which begins at the amino terminal Glu1 residue of the variable region through to Ala 117. The murine LM609 antibody heavy chain has an IgG2a constant region. Shown in Figure 2B is the LM609 light chain variable region polypeptide which is encoded by a nucleic acid of about 321 nucleotides in length which begins at the amino terminal Asp1 residue of the variable region through to Lys 107. In the functional antibody, LM609 has a kappa light chain constant region.

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As with the Vitaxin nucleic acids, minor modifications of these LM609 nucleotide sequences are intended to be included as heavy and light chain LM609 encoding nucleic acids. Such minor modifications are included within the nucleic acids encoding LM609 heavy and light chain polypeptides so long as the nucleic acids or encoded polypeptides retain some or all of their function as described.

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Thus, the invention also provides a nucleic acid encoding a LM609 heavy chain or functional fragment wherein the nucleic acid encodes substantially the same variable region amino acid sequence of monoclonal antibody LM609 as that shown in Figure 2A (SEQ ID NO:6) or a fragment thereof. Similarly, the invention also provides a nucleic acid encoding a LM609 light chain or functional fragment wherein the nucleic acid encodes substantially the same variable region amino acid sequence of monoclonal antibody LM609 as that shown in Figure 2B (SEQ ID NO:8) or a fragment thereof.

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5 The invention further provides fragments of
LM609 heavy and light chain encoding nucleic acids
wherein such fragments consist substantially of the same
10 nucleotide or amino acid sequence as the variable region
5 of LM609 heavy or light chain polypeptides. The variable
region of the LM609 heavy chain polypeptide consists
essentially of nucleotides 1-351 and of amino acid
15 residues Glu1 to Ala117 of Figure 2A (SEQ ID NOS:5 and 6,
respectively). The variable region of the LM609 light
10 chain polypeptide consists essentially of nucleotides
1-321 and of amino acid residues Asp1 to Lys107 of Figure
20 2B (SEQ ID NOS:7 and 8, respectively). The termini of
such variable region encoding nucleic acids is not
critical so long as the intended purpose and function
15 remains the same. Such intended purposes and functions
include, for example, use for the production of
25 recombinant polypeptides or as hybridization probes for
heavy and light chain variable region sequences.

30 Fragments additional to the variable region
20 nucleic acid fragments are provided as well. Such
fragments include, for example, nucleic acids consisting
substantially of the same nucleotide sequence as a CDR of
35 a LM609 heavy or light chain polypeptide. Sequences
corresponding to the LM609 CDRs include, for example,
25 those regions within the variable region which are
defined by Kabat et al., *supra*, and/or those regions
40 within the variable regions which are defined by Chothia
et al., *supra*, as well as those regions defined by
MacCallum et al., *supra*. The LM609 CDR fragments for
45 30 each of the above definitions correspond to the
nucleotides set forth below in Table 4. The nucleotide
sequence numbering is taken from the primary sequence
shown in Figures 2A and 2B (SEQ ID NOS:5 and 7) and
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conforms to the definitions previously set forth in Table 1.

Table 4: LM609 CDR Nucleotide Residues

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V _H CDR1	91-105	76-96	88-105
V _H CDR2	148-198	157-168	139-177
V _H CDR3	295-318	298-315	288-315
V _L CDR1	70-102	76-96	88-108
V _L CDR2	148-168	148-156	136-165
V _L CDR3	265-291	271-288	265-288

Similarly, the LM609 CDR fragments for each of the above definitions correspond to the amino acid residues set forth below in Table 5. The amino acid residue numbering is taken from the primary sequence shown in Figures 2A and 2B (SEQ ID NOS:6 and 8) and conforms to the definitions set forth in Table 1.

Table 5: LM609 CDR Amino Acid Residues

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V _H CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
V _H CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
V _H CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
V _L CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
V _L CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
V _L CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Nucleic acids encoding LM609 heavy and light chain polypeptides and fragments thereof are useful for a variety of diagnostic and therapeutic purposes. For example, the LM609 nucleic acids can be used to produce recombinant LM609 antibodies and functional fragments thereof having binding specificity and inhibitory

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activity against the integrin $\alpha_v\beta_3$. The antibody and functional fragments thereof can be used to determine the presence or absence of $\alpha_v\beta_3$ in a sample to diagnose the susceptibility or occurrence of an $\alpha_v\beta_3$ -mediated disease.

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5 Alternatively, the recombinant LM609 antibodies and functional fragments thereof can be used for the therapeutic treatment of $\alpha_v\beta_3$ -mediated diseases or pathological state. As with Vitaxin, recombinant LM609 and functional fragments thereof can be used to inhibit the binding activity or other functional activities of $\alpha_v\beta_3$ that are necessary for progression of the $\alpha_v\beta_3$ -mediated disease or pathological state.

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The LM609 nucleic acids of the invention can also be used to model functional equivalents of the encoded heavy and light chain polypeptides. Such functional equivalents can include, for example, synthetic analogues or mimics of the encoded polypeptides or functional fragments thereof. A specific example would include peptide mimetics of the LM609 CDRs that retain some or substantially the same binding or inhibitory activity of LM609. Additionally, the LM609 encoding nucleic acids can be used to engineer and produce nucleic acids which encode modified forms or derivatives of the antibody LM609, its heavy and light chain polypeptides and functional fragments thereof. As described previously, such modified forms or derivatives include, for example, non-mouse antibodies, their corresponding heavy and light chain polypeptides and functional fragments thereof which exhibit substantially the same binding and inhibitory activity as LM609.

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The invention also provides a method of treating an $\alpha_v\beta_3$ -mediated disease. The method consists of administering an effective amount of Vitaxin, a LM609

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5 grafted antibody, an enhanced antibody thereof, or a
functional fragment thereof under conditions which allow
binding to $\alpha_v\beta_3$. Also provided is a method of inhibiting
10 a function of $\alpha_v\beta_3$. The method consists of contacting $\alpha_v\beta_3$
5 with Vitaxin, a LM609 grafted antibody or a functional
fragment thereof under conditions which allow binding to
 $\alpha_v\beta_3$.

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As described previously, Vitaxin and LM609
grafted antibodies are monoclonal antibodies which
10 exhibit essentially all of the binding characteristics as
20 does its parental CDR-donor antibody LM609. These
characteristics include, for example, significant binding
specificity and affinity for the integrin $\alpha_v\beta_3$. The
25 Examples below demonstrate these binding properties and
15 further show that the binding of such antibodies to $\alpha_v\beta_3$
inhibits $\alpha_v\beta_3$ ligand binding and function. Thus, Vitaxin
and LM609 grafted antibodies are useful for a large
variety of diagnostic and therapeutic purposes directed
30 to the inhibition of $\alpha_v\beta_3$ function.

20 The integrin $\alpha_v\beta_3$ functions in numerous cell
35 adhesion and migration associated events. As such, the
dysfunction or dysregulation of this integrin, its
function, or of cells expressing this integrin, is
associated with a large number of diseases and
40 25 pathological conditions. The inhibition $\alpha_v\beta_3$ binding or
function can therefore be used to treat or reduce the
severity of such $\alpha_v\beta_3$ -mediated pathological conditions.
Described below are examples of several pathological
45 conditions mediated by $\alpha_v\beta_3$, since the inhibition of at
30 least this integrin reduces the severity of the
condition. These examples are intended to be
representative and as such are not inclusive of all
50 $\alpha_v\beta_3$ -mediated diseases. For example, there are numerous

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pathological conditions additional to those discussed below which exhibit the dysregulation of $\alpha_v\beta_3$ binding, function or the dysregulation of cells expressing this integrin and in which the pathological condition can be reduced, or will be found to be reduced, by inhibiting the binding $\alpha_v\beta_3$. Such pathological conditions which exhibit this criteria, are intended to be included within the definition of the term as used herein.

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Angiogenesis, or neovascularization, is the process where new blood vessels form from pre-existing vessels within a tissue. As described further below, this process is mediated by endothelial cells expressing $\alpha_v\beta_3$ and inhibition of at least this integrin, inhibits new vessel growth. There are a variety of pathological conditions that require new blood vessel formation or tissue neovascularization and inhibition of this process inhibits the pathological condition. As such, pathological conditions that require neovascularization for growth or maintenance are considered to be $\alpha_v\beta_3$ -mediated diseases. The extent of treatment, or reduction in severity, of these diseases will therefore depend on the extent of inhibition of neovascularization. These $\alpha_v\beta_3$ -mediated diseases include, for example, inflammatory disorders such as immune and non-immune inflammation, chronic articular rheumatism, psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma and capillary proliferation in atherosclerotic plaques as well as cancer disorders. Such cancer disorders can include, for example, solid tumors, tumor metastasis, angiofibromas, retrolental, fibroplasia, hemangiomas, Kaposi's sarcoma and other cancers which require neovascularization to support tumor growth. Additional diseases which are considered

5 angiogenic include psoriasis and rheumatoid arthritis as
well as retinal diseases such as macular degeneration.
Diseases other than those requiring new blood vessels
10 which are $\alpha_v\beta_3$ -mediated diseases include, for example,
5 restenosis and osteoporosis.

Treatment of the $\alpha_v\beta_3$ -mediated diseases can be
15 performed by administering an effective amount of
Vitaxin, a LM609 grafted antibody, an enhanced antibody
thereof, or a functional fragment thereof so as to bind
10 to $\alpha_v\beta_3$ and inhibit its function. Administration can be
20 performed using a variety of methods known in the art.
The choice of method will depend on the specific $\alpha_v\beta_3$ -
mediated disease and can include, for example, the *in*
25 *vivo*, *in situ* and *ex vivo* administration of Vitaxin, a
15 LM609 grafted antibody or functional fragment thereof, to
cells, tissues, organs, and organisms. Moreover, such
antibodies or functional fragments can be administered to
30 an individual exhibiting or at risk of exhibiting an $\alpha_v\beta_3$ -
mediated disease. Definite clinical diagnosis of an $\alpha_v\beta_3$ -
20 mediated disease warrants the administration of Vitaxin,
a LM609 grafted antibody or a functional fragment
35 thereof. Prophylactic applications are warranted in
diseases where the $\alpha_v\beta_3$ -mediated disease mechanisms
precede the onset of overt clinical disease. Thus,
25 individuals with familial history of disease and
40 predicted to be at risk by reliable prognostic indicators
can be treated prophylactically to interdict $\alpha_v\beta_3$ -mediated
mechanisms prior to their onset.

45 Vitaxin, a LM609 grafted antibody, an enhanced
30 antibody thereof, or functional fragments thereof can be
administered in a variety of formulations and
pharmaceutically acceptable media for the effective
50 treatment or reduction in the severity of an $\alpha_v\beta_3$ -mediated

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disease. Such formulations and pharmaceutically acceptable medias are well known to those skilled in the art. Additionally, Vitaxin, a LM609 grafted antibody or functional fragments thereof can be administered with other compositions which can enhance or supplement the treatment or reduction in severity of an $\alpha_v\beta_3$ -mediated disease. For example, the coadministration of Vitaxin or a LM609 grafted antibody to inhibit tumor-induced neovascularization and a chemotherapeutic drug to directly inhibit tumor growth is one specific case where the administration of other compositions can enhance or supplement the treatment of an $\alpha_v\beta_3$ -mediated disease.

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Vitaxin, a LM609 grafted antibody or functional fragments are administered by conventional methods, in dosages which are sufficient to cause the inhibition of $\alpha_v\beta_3$ integrin binding at the site of the pathology. Inhibition can be measured by a variety of methods known in the art such as *in situ* immunohistochemistry for the prevalence of $\alpha_v\beta_3$ containing cells at the site of the pathology as well as include, for example, the observed reduction in the severity of the symptoms of the $\alpha_v\beta_3$ -mediated disease.

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In vivo modes of administration can include intraperitoneal, intravenous and subcutaneous administration of Vitaxin, a LM609 grafted antibody or a functional fragment thereof. Dosages for antibody therapeutics are known or can be routinely determined by those skilled in the art. For example, such dosages are typically administered so as to achieve a plasma concentration from about 0.01 $\mu\text{g/ml}$ to about 100 $\mu\text{g/ml}$, preferably about 1-5 $\mu\text{g/ml}$ and more preferably about 5 $\mu\text{g/ml}$. In terms of amount per body weight, these dosages typically correspond to about 0.1-300 mg/kg, preferably

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5 about 0.2-200 mg/kg and more preferably about 0.5-20
mg/kg. Depending on the need, dosages can be
administered once or multiple times over the course of
10 the treatment. Generally, the dosage will vary with the
5 age, condition, sex and extent of the $\alpha_v\beta_3$ -mediated
pathology of the subject and should not be so high as to
cause adverse side effects. Moreover, dosages can also
15 be modulated by the physician during the course of the
treatment to either enhance the treatment or reduce the
10 potential development of side effects. Such procedures
are known and routinely performed by those skilled in the
20 art.

The specificity and inhibitory activity of
25 Vitaxin, LM609 grafted antibodies, an enhanced antibody
15 thereof and functional fragments thereof allow for the
therapeutic treatment of numerous $\alpha_v\beta_3$ -mediated diseases.
Such diseases include, for example, pathological
30 conditions requiring neovascularization such as tumor
growth, and psoriasis as well as those directly mediated
20 by $\alpha_v\beta_3$, such as restenosis and osteoporosis. Thus, the
invention provides methods as well as Vitaxin and LM609
35 grafted antibody containing compositions for the
treatment of such diseases.

It is understood that modifications which do
40 25 not substantially affect the activity of the various
embodiments of this invention are also included within
the definition of the invention provided herein.
Accordingly, the following examples are intended to
45 illustrate but not limit the present invention.

EXAMPLE I

Isolation and Characterization of LM609Encoding Nucleic Acids

This Example shows the cloning and sequence determination of LM609 encoding nucleic acids.

LM609 is directed against the human vitronectin receptor, integrin $\alpha_v\beta_3$. $\alpha_v\beta_3$ is highly upregulated in melanoma, glioblastoma, and mammary carcinoma and plays a role in the proliferation of M21 melanoma cells both in vitro and in vivo. $\alpha_v\beta_3$ also plays a role in angiogenesis, restenosis and the formation of granulation tissue in cutaneous wounds. LM609 has been shown to inhibit the adhesion of M21 cells to vitronectin as well as prevent proliferation of M21 cells in vitro. Thus, grafting of LM609 could result in a clinically valuable therapeutic agent.

cDNA Synthesis of LM609 Variable Regions: For cDNA synthesis, total RNA was prepared from 10^8 LM609 hybridoma cells using a modification of the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, Analyt. Biochem. 162:156 (1987)). LM609 variable (V) region genes were cloned by reverse transcription-polymerase chain reaction (RT-PCR) and cDNA was synthesized using BRL Superscript kit. Briefly, 5 mg of total cellular RNA, 650 ng oligo dT and H_2O were brought to a total volume of 55 μ l. The sample was heated to 70°C for 10 min and chilled on ice. Reaction buffer was added and the mixture brought to 10 mM DTT and 1 mM dNTPs and heated at 37°C for 2 minutes. 5 μ l (1000 units) reverse transcriptase was added and incubated at 37°C for 1 hour and then chilled on ice.

5 All oligonucleotides were synthesized by
β-cyanoethyl phosphoramidite chemistry on an ABI 394 DNA
synthesizer. Oligonucleotides used for PCR amplification
10 and routine site-directed mutagenesis were purified using
5 oligonucleotide purification cartridges (Applied
Biosystems, Foster City, CA). Forward PCR primers were
designed from N-terminal protein sequence data generated
15 from purified LM609 antibody. The forward PCR primers
contained sequences coding for the first six amino acids
10 in each antibody variable chain (protein sequenced at San
Diego State University). The sequence of the light chain
20 forward PCR primer (997) was 5'-GCC CAA CCA GCC ATG GCC
GAT ATT GTG CTA ACT CAG-3' (SEQ ID NO:19) whereas the
light chain reverse PCR primer (734) was 5'-AC AGT TGG
15 TGC AGC ATC AGC-3' (SEQ ID NO:20) used. This reverse
primer corresponds to mouse light chain kappa amino acid
residues 109-115. The sequence of the heavy chain
forward PCR primer (998) was 5'-ACC CCT GTG GCA AAA GCC
30 GAA GTG CAG CTG GTG GAG-3' (SEQ ID NO:21). Heavy chain
20 reverse PCR primer 733: 5'-GA TGG GGG TGT CGT TTT GGC-3'
(SEQ ID NO:22). The PCR primers also contain regions of
homology with specific sequences within the
35 immunoexpression vector.

V_L and V_H chains were amplified in two separate
25 50 ml reaction mixtures containing 2 ml of the cDNA-RNA
heteroduplex, 66.6 mM Tris-HCl pH 8.8, 1.5 mM MgCl₂, 0.2
40 mM of each four dNTPs, 10 mM 2-mercaptoethanol, 0.25
units Taq polymerase (Boehringer-Mannheim, Indianapolis,
IN) and 50 pmoles each of primers 997 and 734 and 998 and
45 733, respectively. The mixtures were overlaid with
30 mineral oil and cycled for two rounds of PCR with each
cycle consisting of 30 seconds at 94°C (denature), 30
seconds at 50°C (anneal), and 30 seconds at 72°C
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5 (synthesis). This reaction was immediately followed by
30 cycles of PCR consisting of 30 seconds at 94°C
10 (denature), 30 seconds at 55°C (anneal), and 30 seconds at
72°C (synthesis) followed by a final synthesis reaction
5 for 5 minutes at 72°C. The reaction products were pooled,
extracted with CHCl₃, and ethanol precipitated.

15 Amplified products were resuspended in 20 ml TE
buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and
electrophoresed on a 5% polyacrylamide gel. Bands
10 migrating at expected molecular weights of V_H and V_L were
excised, chemically eluted from the gel slice, extracted
20 with organic solvents and ethanol precipitated.

25 Cloning of amplified V_H and V_L genes into M13
phage immunoexpression vector: The amplified V region
15 gene products were sequentially cloned into the phage
immunoexpression vector by hybridization mutagenesis
(Near, R. Biotechniques 12:88 (1992); Yelton et al., J.
30 Immunol. 155:1994-2003 (1995)). Introduction of the
amplified V_L and V_H sequences by hybridization mutagenesis
20 positions the antibody sequences in frame with the
regulatory elements contained in the M13 vector required
35 for efficient Fab expression. One advantage of this
technique is that no restriction endonuclease sites need
to be incorporated into the V_L or V_H gene sequences for
40 25 cloning as is done with conventional DNA ligation
methods.

45 To perform the cloning, 400 ng each of the
double-stranded amplified products were first
phosphorylated with polynucleotide kinase. 100 ng of the
30 phosphorylated LM609 V_L product was mixed with 250 ng of
uridinylated BS11 phage immunoexpression vector,
50 denatured by heating to 90°C and annealed by gradual

5 cooling to room temperature. BS11 is an M13
immunoexpression vector derived from M13 IX and encodes
CH₁ of murine IgG1 and murine kappa light chain constant
10 domain (Huse, W.D. In: Antibody Engineering: A Practical
5 Guide, C.A.K. Borrebaeck, ed. W.H. Freeman and Co.,
Publishers, New York, pp. 103-120 (1991)). Nucleotide
sequences included in the PCR amplification primers
15 anneal to complementary sequences present in the single-
stranded BS11 vector. The annealed mixture was fully
10 converted to a double-stranded molecule with T4 DNA
polymerase plus dNTPs and ligated with T4 ligase. 1 ml
20 of the mutagenesis reaction was electroporated into *E.*
coli strain DH10B, titered onto a lawn of XL-1 *E. coli*
and incubated until plaques formed. Plaque lift assays
25 were performed as described using goat anti-murine kappa
chain antibody conjugated to alkaline phosphatase (Yelton
et al, *supra*; Huse, W.D., *supra*). Fifteen murine light
chain positive M13 phage clones were isolated, pooled and
30 used to prepare uridynylated vector to serve as template
20 for hybridization mutagenesis with the PCR amplified
LM609 V_H product.

35 Clones expressing functional murine LM609 Fab
were identified by binding to purified a₂b₂ by ELISA.
Briefly, Immulon II ELISA plates were coated overnight
25 with 1 mg/ml (100 ng/well) a₂b₂ and nonspecific sites
40 blocked for two hours at 27°C. Soluble Fabs were prepared
by isolating periplasmic fractions of cultures of *E. coli*
strain MK30-3 (Boehringer Mannheim Co.) infected with the
Fab expressing M13 phage clones. Periplasm fractions
45 30 were mixed with binding buffer 100 mM NaCl, 50 mM Tris pH
7.4, 2mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 0.02% NaN₃, 1 mg/ml
BSA and incubated with immobilized a₂b₂ for two hours at
50 27°C. Plates were washed with binding buffer and bound

5 Fab detected with goat anti-murine kappa chain antibody
conjugated to alkaline phosphatase. Four a₁b₂ reactive
clones were identified: muLM609M13 12, 29, 31 and 69.
10 MuLM609M13 12 and 29 gave the strongest signals in the
5 ELISA assay. DNA sequence analysis showed that clones
muLM609M13 12, 31 and 69 all had identical light chain
sequence and confirmed the previously determined
15 N-terminal amino acid sequence of purified LM609 light
chain polypeptide. All four clones had identical V_H DNA
10 sequence and also confirmed the previously determined
N-terminal amino acid sequence of purified LM609 heavy
20 chain polypeptide.

To further characterize the binding activity of
each clone, soluble Fab fractions were prepared from 50
25 ml cultures of *E. coli* strain MK30-3 infected with clones
12 and 29 and evaluated for binding to a₁b₂ in a
competitive ELISA with LM609 IgG. The results of this
30 ELISA are shown in Figure 3. Clone muLM609M13 12 was
found to inhibit LM609 IgG binding (at LM609 IgG
20 concentrations of 1 ng/ml and 5 ng/ml) to a₁b₂ in a
concentration dependent manner at periplasm titers
ranging from neat to 1:80. Clone muLM609M13 12 was
35 plaque purified and both the V region heavy and light
chain DNA sequences again determined. Complete DNA
25 sequence of the final clone, muLM609M13 12-5, is shown in
40 Figures 2A and 2B.

EXAMPLE II

Construction of Vitaxin: A CDR Grafted

LM609 Functional Fragment

30 One goal of grafting antibodies is to preserve
antibody specificity and affinity when substituting
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non-human CDRs into a human antibody framework. Another goal is to minimize the introduction of foreign amino acid sequences so as to reduce the possible antigenicity with a human host. This Example describes procedures for accomplishing both of these goals by producing libraries of grafted antibodies which represent all possible members which exhibit the highest affinities for the desired antigen.

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The above library was constructed in *E. coli* wherein the possible CDR and framework changes were incorporated using codon-based mutagenesis (Kristensson et al., In: Vaccines 95. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY (1995); Rosok et al., J. Biol. Chem. (271:22611-22613 (1996)). Using these procedures, a library was constructed and a functionally active humanized anti- $\alpha\beta_3$ -inhibitory antibody was identified.

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For the construction of one grafted form of LM609, human framework sequences showing the highest degree of identity to the murine LM609 V region gene sequences were selected for receiving the LM609 CDRs. Human heavy chain V region M72 'CL (HHC30Q, HC Subgroup 3, Kabat et al., *supra*) had 88% identity to frameworks 1, 2 and 3 of LM609 heavy chain and human light chain V region LS1 'CL (HKL312, Kappa subgroup 3, Kabat et al., *supra*) had 79% identity to frameworks 1, 2 and 3 of LM609 light chain. Murine LM609 CDR sequences, as defined by Kabat et al., *supra* were grafted onto the human frameworks. Residues predicted to be buried that might affect the structure and therefore the binding properties of the original murine combining site were taken into consideration when designing possible changes (Singer et al., *supra*; Padlan, E.A. Mol. Immunol. 28:489-498

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(1991)). This analysis of framework residues considered to be important for preserving the specificity and affinity of the combining site revealed only a few differences. For example, in the heavy chain sequence, the predicted buried residues displayed 100% identity. Of particular note is that Arg16 in human heavy chain V region M72 'CL is a relatively uncommon residue among human chains. However, this residue was also found to be present in LM609 V_H and therefore was retained. Similarly, Arg19 in LM609 is a relatively rare residue among murine heavy chains but it is found to occur in M72 'CL and was therefore retained. In the light chain sequences, two nonidentical buried residues were identified between LM609 and LS1 'CL framework regions at positions 49 and 87. These two positions were therefore incorporated into the grafted antibody library as both human and murine alternatives.

Full-length grafted V region genes were synthesized by PCR using long overlapping oligonucleotides. Light chain oligonucleotides containing mixed amino acid residues at positions 49 and 87 were synthesized as described in Glaser et al. (J. Immunol. 149:3903-3913 (1992)) and as illustrated in the oligonucleotides represented as V_L oligo3 and V_L oligo4. (SEQ ID NOS:16 and 17, respectively). All long oligonucleotides were gel purified.

Grafted LM609 heavy and light chain V regions were constructed by mixing 5 overlapping oligonucleotides at equimolar concentrations, in the presence of annealing PCR primers. The heavy chain oligonucleotides map to the following nucleotide positions: V_H oligonucleotide 1 (V_H oligo1), nucleotides (nt) 1-84; (SEQ ID NO:9); V_H oligo2, nt 70-153, (SEQ ID NO:10); V_H oligo3, nt 138-225 (SEQ ID

5 NO:11); V_H oligo4, nt 211-291 (SEQ ID NO:12); V_H oligo5,
nt 277-351 (SEQ ID NO:13). Similarly, the Vitaxin light
chain oligonucleotides map to the following nucleotide
10 positions: V_L oligonucleotide 1 (V_L oligo1), nucleotides
5 (nt) 1-87; (SEQ ID NO:14); V_L oligo2, nt 73-144, (SEQ ID
NO:15); V_L oligo3, nt 130-213 (SEQ ID NO:16); V_L oligo4,
nt 199-279 (SEQ ID NO:17); V_L oligo5, nt 265-321 (SEQ ID
15 NO:18). The nucleotide sequences of oligonucleotides
used to construct grafted LM609 heavy and light chain
10 variable regions are shown in Table 6. Codon positions
49 and 87 in V_L oligo3, and V_L oligo4 represent the
20 randomized codons. The annealing primers contained at
least 18 nucleotide residues complementary to vector
sequences for efficient annealing of the amplified V
15 region product to the single-stranded vector. The
25 annealed mixture was fully converted to a double-stranded
molecule with T4 DNA polymerase plus dNTPs and ligated
with T4 ligase.

30 To generate the library, a portion of the
20 mutagenesis reaction (1 μ l) was electroporated into *E.*
coli strain DH10B (BRL), titered onto a lawn of XL-1
(Stratagene, Inc.) and incubated until plaques formed.
35 Replica filter lifts were prepared and plaques containing
V_H gene sequences were screened either by hybridization
25 with a digoxigenin-labeled oligonucleotide complementary
40 to LM609 heavy chain CDR 2 sequences or reactivity with
7F11-alkaline phosphatase conjugate, a monoclonal
antibody raised against the decapeptide sequence Tyr Pro
Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended
45 30 to the carboxy terminus of the vector CH₁ domain (Biosite,
Inc., San Diego, CA). Fifty clones that were double-
positive were pooled and used to prepare uridynylated
50 template for hybridization mutagenesis with the amplified
grafted LM609 V_L product.

**Table 6: Oligonucleotides Used to Construct Grafted
LM609 Heavy and Light Chain Variable Regions**

	CAGGTGCAGC	TGGTGGAGTC	TGGGGGAGGC	GTTGTGCAGC	CTGGAAGGTC	
10	CCTGAGACTC	TCCTGTGCAG	CCTCTGGATT	CACC	SEQ ID NO: 9	
5	AACTTTTGC	ACCCACTCCA	GACCCTTGCC	CGGAGCCTGG	CGAACCCAAG	
	ACATGTCATA	GCTACTGAAG	GTGAATCCAG	AGGC	SEQ ID NO: 10	
	TGGGTCGCAA	AAGTTAGTAG	TGGTGGTGGT	AGCACCTACT	ATTAGACAC	
15	TGTGCAGGGC	CGATTCACCA	TCTCCAGAGA	CAATAGT	SEQ ID NO: 11	
	TGCACAGTAA	TACACGGCTG	TGTCCTCGGC	TCTCAGAGAG	TTCATTTGCA	
10	GGTATAGGGT	GTTCTTACTA	TTGTCTCTGG	A	SEQ ID NO: 12	
	GTGTATTACT	GTGCAAGACA	TAACACGGC	AGTTTGTGCTT	ACTGGGGCCA	
20	AGGGACTACA	GTGACTGTTT	CTAGT		SEQ ID NO: 13	
	GAGATTGTGC	TAACACAGTC	TCCAGCCACC	CTGTCTCTCA	GCCCAGGAGA	
	AAGGGCGACT	CTTTCCTGCC	AGGCCAGCCA	AAGTATT	SEQ ID NO: 14	
25	GATGAGAAGC	CTTGGGGCTT	GACCAGGCCT	TTGTTGATAC	CAGTGTAGGT	
	GGTTGCTAAT	ACTTTGGCTG	GC		SEQ ID NO: 15	
	CCAAGGCTTC	TCATCWAATA	TCGTTCCAG	TCCATCTCTG	GGATCCCCGC	
	CAGGTTCACT	GGCAGTGGAT	CAGGGACAGA	TTTC	SEQ ID NO: 16	
30	GCTGCCACTC	TGTTGACAGW	AATAGACTGC	AAAATCTTCA	GGCTCCAGAC	
20	TGGAGATAGT	GAGGGTGAAA	TCTGTCCCTG	A	SEQ ID NO: 17	
	CAACAGAGTG	GCAGCTGGCC	TCACACGTTT	GGAGGGGGGA	CCAAGGTGGA	
	AATTAAG				SEQ ID NO: 18	

The mutagenesis reaction was performed as described above with the V_H oligonucleotides except that the V_L oligonucleotides 1 to 5 were employed (SEQ ID NOS:14 to 18, respectively). The reaction was electroporated into *E. coli* strain DH10B and filter lifts probed with either goat anti-human kappa chain antibody conjugated to alkaline phosphatase or a goat anti-human Fab antibody using an alkaline phosphatase conjugated rabbit anti-goat secondary reagent for detection. Positive clones co-expressing both V_H and V_L gene sequences were selected (160 total) and used to infect *E. coli* strain MK30-3 for preparing soluble Fab fragments.

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The soluble Fab fragments were screened for binding to $\alpha_v\beta_3$ in an ELISA assay. Four clones that were shown from the ELISA to strongly bind $\alpha_v\beta_3$ were identified and further characterized. These clones were termed huLM609M13-34, 54, 55 and 145. All four clones were plaque purified and three independent subclones from each clone was used to prepare Fab fragments for additional binding analysis to $\alpha_v\beta_3$ by ELISA.

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In this additional ELISA, duplicate plates were coated with $\alpha_v\beta_3$ ligand and incubated with the huLM609 periplasmic samples. In one plate, bound huLM609 Fab was detected with goat anti-human kappa chain antibody conjugated to alkaline phosphatase and in the other plate bound huLM609 Fab was detected with 7F11-alkaline phosphatase conjugate, the monoclonal antibody recognizing the decapeptide tag. Subclones huLM609M13-34-1, 2 and 3 and huLM609M13-145-1, 2 and 3 all yielded double positive signals indicating that the Fabs contain functional V_H and V_L polypeptides. These results were confirmed in an ELISA assay on M21 cells, a cell line that expresses the integrin $\alpha_v\beta_3$.

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DNA sequence analysis of subclones huLM609M13-34-3 and huLM609M13-145-3 revealed mutations introduced into the library by errors due to oligonucleotide synthesis or by errors arising during PCR amplification. These mutations were corrected in clone huLM609M13-34-3 by site-directed mutagenesis. In the light chain sequence the following corrections were made: His36 to Tyr36 and Lys18 to Arg18. In the heavy chain sequence the following corrections were made: Glu1 to Gln1, Asn3 to Gln3, Leu11 to Val11. Additionally, during the construction of LM609 grafted molecules, residue 28 from

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the heavy chain was considered to be a non-critical framework residue and the human residue (Thr28) was retained. Subsequently, however, it has been determined that residue 28 can be considered part of the CDR.

Therefore, residue 28 was converted to the corresponding mouse residue at that position (Ala28) using site directed mutagenesis with the oligonucleotide 5'-GCT ACT GAA GGC GAA TCC AGA G-3' (SEQ ID NO:29). This change was later determined to not provide benefit over the human framework threonine at this site, and the threonine was retained. The final grafted LM609 clone was designated huLM609M13 1135-4 and is termed herein Vitaxin. The DNA sequence of clone Vitaxin is shown in Figures 2A and 2B.

EXAMPLE III

Functional Characterization of Vitaxin

This Example shows the characterization of Vitaxin's binding specificity, affinity and functional activity in a number of *in vitro* binding and cell adhesion assays.

The binding specificity of Vitaxin for the integrin $\alpha_v\beta_3$ was initially assessed by measuring binding to $\alpha_v\beta_3$ and its crossreactivity to other α_v - or β_3 -containing integrins. Specifically, binding specificity was assessed by measuring binding to $\alpha_{IIb}\beta_3$, the major integrin expressed on platelets, and to $\alpha_v\beta_5$, an integrin found prevalent on endothelial cells and connective tissue cell types.

Briefly, to determine crossreactivity, integrins were coated onto an ELISA plate and a series of antibody dilutions were measured for Vitaxin binding activity against $\alpha_v\beta_3$ and the other integrins. The

5 integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were isolated by affinity
chromatography as described by Cheresh (1987), *supra*, and
10 Cheresh and Spiro (1987), *supra*. $\alpha_{IIB}\beta_3$ was purchased from
CalBiochem. Briefly, an LM609 affinity column (Cheresh
5 and Spiro (1987), *supra*) was used to isolate $\alpha_v\beta_3$ from an
octylglucoside human placental lysate, whereas an anti- α_v
15 affinity column was used to isolate $\alpha_v\beta_5$ from the
 $\alpha_v\beta_3$ -depleted column flow through. Antibody binding
activity was assessed by ELISA using a goat anti-human
10 IgG-alkaline phosphatase conjugate. As a control, a
purified human IgG₁ antibody was used since Vitaxin
20 contains a human IgG₁ backbone.

The results of this assay are shown in Figure
25 4A and reveal that Vitaxin specifically binds to $\alpha_v\beta_3$ with
15 high affinity. There was no detectable binding to the
other α_v - or β_3 -containing integrins at antibody
concentrations over 1.0 mg/ml.

30 In a further series of binding studies, the
binding affinity and specificity was assessed in a
20 competitive binding assay with the parental LM609
antibody against $\alpha_v\beta_3$. Competitive binding was measured
35 in an ELISA assay as described above with LM609 being the
labeled antibody. Binding of LM609 was determined in the
presence of increasing concentrations of Vitaxin
40 25 competitor. Alternatively, the control competitor
antibody was again a human IgG₁.

45 The results of this competition are presented
in Figure 4B and show that specific inhibition of LM609
binding can be observed at Vitaxin concentrations of over
30 0.1 μ g/ml. Almost complete inhibition is observed at
Vitaxin concentrations greater than 100 μ g/ml. This
50 level of competitive inhibition indicates that the

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parental monoclonal antibody LM609 and the grafted version Vitaxin exhibit essentially identical specificity.

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Binding affinity and specificity were also assessed by measuring the inhibitory activity of Vitaxin on $\alpha_v\beta_3$ binding to fibrinogen. For these studies, $\alpha_v\beta_3$ was plated onto ELISA plates as described above for the Vitaxin/ $\alpha_v\beta_3$ binding studies. Inhibitory activity of Vitaxin was determined by measuring the amount of bound biotinylated fibrinogen in the presence of increasing concentrations of Vitaxin or control antibody. Briefly, fibrinogen was purchased from CalBiochem and biotinylated with N-hydroxysuccinimidobiotin as described by the manufacturer (Pierce Life Science and Analytical Research). Streptavidin alkaline phosphatase was used to detect the bound fibrinogen.

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The results of this assay are presented in Figure 4C and reveal a specific binding inhibition at Vitaxin concentrations higher than about 0.1 $\mu\text{g/ml}$. These results, combined with those presented above showing specific binding of Vitaxin to $\alpha_v\beta_3$ and competitive inhibition of LM609, demonstrate that Vitaxin maintains essentially all of the binding characteristics and specificity exhibited by the parental murine monoclonal antibody LM609. Described below are additional functional studies which corroborate these conclusions based on *in vitro* binding assays.

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Additional functional studies were performed to further assess the specificity of Vitaxin binding. These studies were directed to the inhibition of integrin $\alpha_v\beta_3$ binding in cell adhesion assays. Endothelial cell adhesion events are an important component in the

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angiogenic process and inhibition of $\alpha_v\beta_3$ is known to reduce the neovascularization of tumors and thereby reduce the rate of tumor growth. The inhibition of $\alpha_v\beta_3$ -mediated cell attachment by Vitaxin in these assays is indicative of the inhibitory activity expected when this antibody is used *in situ* or *in vivo*.

Briefly, $\alpha_v\beta_3$ -positive M21 melanoma cells grown in RPMI containing 10% FBS were used for these cell binding assays. Cells were released from the culture dish by trypsinization and re-suspended in adhesion buffer at a concentration of 4×10^5 cells/ml (see below). Vitaxin, LM609 or purified human IgG₁ (control antibody), were diluted to the desired concentration in 250 μ l adhesion buffer (10 mM Hepes, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mM MnCl₂, and 1% BSA in Hepes buffered saline at pH 7.4) and added to wells of a 48-well plate precoated with fibrinogen. The fibrinogen was isolated as described above. Each well was coated with 200 μ l fibrinogen at a concentration of 10 μ g/ml for 1 hour at 37°C. For the assay, an equal volume of cells (250 μ l) containing Vitaxin, LM609 or isotype matched control antibody was added to each of the wells, mixed by gentle shaking and incubated for 20 minutes at 37°C. Unbound cells were removed by washing with adhesion buffer until no cells remained in control wells coated with BSA alone. Bound cells were visualized by staining with crystal violet which was subsequently extracted with 100 μ l acetic acid (10%) and quantitated by determining the absorbance of the solubilized dye at 560 nm.

The results of this assay are shown in Figure 5A and reveal that both Vitaxin and parental antibody LM609 inhibit M21 cell adhesion to fibrinogen over the same concentration range. The inhibitory concentration

5 for 50% maximal adhesion was calculated to be about 50
ng/ml. Specificity of Vitaxin was shown by the lack of
inhibition observed by the control IgG₁ antibody.

10 In addition to the above cell adhesion results,
5 the inhibitory activity of Vitaxin was also tested in an
endothelial cell migration assay. In this regard, the
15 transwell cell migration assay was used to assess the
ability of Vitaxin to inhibit endothelial cell migration
(Choi et al., J. Vascular Surg., 19:125-134 (1994) and
10 Leavesly et al., J. Cell Biol., 121:163-170 (1993)).

20 Briefly, human umbilical vein endothelial cells
in log phase and at low passage number were harvested by
gentle trypsinization, washed and resuspended at a
25 concentration of 2×10^6 cells/ml in 37°C HBS containing
15 1% BSA (20 mM HEPES, 150 mM NaCl, 1.8 mM CaCl₂, 1.8 mM
MgCl₂, 5 mM KCl, and 5 mM glucose, pH 7.4). Antibodies
(Vitaxin, LM609, and IgG₁ control) were diluted to 10
30 µg/ml from stock solutions. Antibodies were added to
cells in a 1:1 dilution (final concentration of
20 antibodies = 5 µg/ml; final concentration of cells = $1 \times$
35 10^6 cells/ml) and incubated on ice for 10 - 30 minutes.
The cell/antibody suspensions (200 µl to each
compartment) were then added to the upper compartments of
a Transwell cell culture chamber (Corning Costar), the
40 lower compartments of which had been coated with 0.5 ml
25 of 10 µg/ml vitronectin (in HBS). Vitronectin serves as
the chemoattractant for the endothelial cells. The
chambers were placed at 37°C for 4 hours to allow cell
45 migration to occur.

30 Visualization of cell migration was performed
by first removing the remaining cells in the upper
50 compartment with a cotton swab. Cells that had migrated

5 to the lower side of the insert were stained with crystal
violet for 30 minutes, followed by solubilization in
10 acetic acid and the absorbance of the dye was measured at
5 directly proportional to the number of cells that have
migrated from the upper to the lower chamber. The
15 results of the assay are presented in Figure 7B. Both
Vitaxin and the parental antibody LM609 yielded
essentially identical inhibitory results. Specifically,
10 Vitaxin and LM609 inhibited about 60% of the vitronectin-
induced migration of endothelial cells compared to the
20 IgG₁ control and to a sample with no inhibitor.

EXAMPLE IV

Vitaxin-Mediated Inhibition of $\alpha_v\beta_3$ In Animal Models

15 This Example describes the inhibition of tumor
growth by Vitaxin in two animal models. Tumor growth was
30 inhibited by inhibiting at least $\alpha_v\beta_3$ -mediated
neovascularization with Vitaxin.

35 The first model measures angiogenesis in the
20 chick chorioallantoic membrane (CAM). This assay is a
well recognized model for *in vivo* angiogenesis because
the neovascularization of whole tissue is occurring.
Specifically, the assay measures growth factor induced
40 angiogenesis of chicken CAM vessels growing toward the
25 growth factor-impregnated filter disk or into the tissue
grown on the CAM. Inhibition of neovascularization is
based on the amount and extent of new vessel growth or on
45 the growth inhibition of tissue on the CAM. The assay
has been described in detail by others and has been used
30 to measure neovascularization as well as the
neovascularization of tumor tissue (Ausprunk et al., Am.
50 J. Pathol., 79:597-618 (1975); Ossonski et al. Cancer

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Res., 40:2300-2309 (1980); Brooks et al. Science, 264:569-571 (1994a) and Brooks et al. Cell, 79:1157-1164 (1994b).

Briefly, for growth factor induced angiogenesis 5 filter disks are punched from #1 Whatman Qualitative Circles using a skin biopsy punch. Disks are first 15 sterilized by exposure to UV light and then saturated with varying concentrations of TNF- α or HBSS as a negative control (for at least 1 hour) under sterile 10 conditions. Angiogenesis is induced by placing the saturated filter disks on the CAMs.

Inhibition of angiogenesis is performed by 25 treating the embryos with various amounts of Vitaxin and controls (antibody or purified human IgG₁). The 15 treatments are performed by intravenous injection approximately 24 hours after disk placement. After 48 30 hours, CAMs are dissected and angiogenesis is scored on a scale of 1-4. HBSS saturated filter disks are used as the negative control, representing angiogenesis that may 20 occur in response to tissue injury in preparing CAMs, and, values for these CAMs are subtracted out as 35 background. Purified human IgG₁ is used as the negative control for injections since Vitaxin is of the human IgG₁ subclass. Vitaxin was found to inhibit TNF- α induced 25 angiogenesis in a dose dependent manner. Maximal inhibition occurred with a single dose of Vitaxin at 300 μ g which resulted in greater than 80% inhibition compared to the human IgG₁ control.

In addition to the above described CAM assay 30 using growth factor-induced neovascularization, additional studies were performed utilizing tumor-induced 50 neovascularization. For these assays, angiogenesis was

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induced by transplanting of $\alpha_v\beta_3$ -negative tumor fragments into the CAMs. The use of $\alpha_v\beta_3$ -negative tumor fragments ensures that any inhibition of tumor growth is due to the inhibition of $\alpha_v\beta_3$ -mediated neovascularization by CAM-derived endothelial cells and not to adhesion events mediated by $\alpha_v\beta_3$ present on the tumor cells.

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Inhibition of tumor growth was assessed by placing a single cell suspension of FG (8×10^6 cells, pancreatic carcinoma) and HEp-3 cells (5×10^5 cells, laryngeal carcinoma) onto CAMs in $30 \mu\text{l}$. One week later, tumors are removed and cut into approximately 50 mg fragments at which time they are placed onto new CAMs. After 24 hours of this second placement embryos are injected intravenously with Vitaxin or human IgG, as a negative control. The tumors are allowed to grow for about 7 days following which they are removed and weighed.

The results of Vitaxin treatment on the neovascularization of tumors is shown in Figure 6A. The data is expressed as a mean change in tumor weight and demonstrate that Vitaxin is able to inhibit the growth of $\alpha_v\beta_3$ -negative tumors such as FG and HEp-3 tumor fragments. More specifically, there was a mean weight change for Vitaxin treated FG tumor fragments of -5.38 whereas a change of -11.0 was observed for Vitaxin treated HEp-3 tumors. The IgG₁ controls exhibited positive mean weight changes of 25.29 and 28.5 for the FG and HEp-3 tumor fragments, respectively. These results were obtained following a single intravenous injection.

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In a second animal model, the inhibition of Vx2 carcinoma cells in rabbits was used as a measure of Vitaxin's inhibitory effect on tumors. The Vx2 carcinoma

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5 is a transplantable carcinoma derived from a Shope
virus-induced papilloma. It was first described in 1940
and has since been used extensively in studies on tumor
10 invasion, tumor-host interactions and angiogenesis. The
5 Vx2 carcinoma is fibrotic in nature, highly aggressive,
and exhibits features of an anaplastic type carcinoma.
Propagation of Vx2 tumor is accomplished through serial
15 transplantation in donor rabbits. Following subcutaneous
transplantation, it has been reported that after an
10 initial inflammatory reaction, host repair mechanisms set
in between days 2 and 4. This repair mechanism is
20 characterized by the formation of new connective tissue
and the production of new capillaries. The newly formed
capillaries are restricted to the repair zone at day 4,
15 however, by day 8 they have extended to the outer region
of the tumor. These characteristics and the
pharmacokinetics of Vitaxin in rabbits were used to
determine initial doses and scheduling of treatments for
30 these experiments. The elimination half life of Vitaxin
20 in animal serum dosed at 1, 5, and 10 mg/kg was found to
be 38.9, 60.3, and 52.1 hours, respectively.

35 Growth of Vx2 tumors in the above animal model
was used to study the effect of Vitaxin after early
administration on primary tumor growth in rabbits
25 implanted subcutaneously with Vx2 carcinoma. Briefly,
40 Vx2 tumors (50 mg) were transplanted into the inner thigh
of rabbits through an incision between the skin and
muscle. Measurements of the primary tumor were taken
throughout the experiment through day 25. At day 28
45 30 after the transplantation animals were sacrificed and
tumors were excised and weighed. By day 28, tumors
became extremely irregular in shape and as a result,
measurements became difficult and were not reflective of
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tumor volume. Therefore measurements were assessed only through day 25.

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In a first study, rabbits were treated starting at day 1 post tumor implantation with 5 and 1 mg/kg Vitaxin every four days for 28 days for a total of 7 doses). In both groups, inhibition of tumor growth was observed. In a second series of studies, rabbits were treated beginning at day 7 post tumor implantation as described above for a total of 5 doses. Inhibition of tumor growth was also observed.

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It should be noted that administering a grafted antibody as a repeat dose treatment to rabbits might generate an immune response that can have a neutralizing effect on Vitaxin thus potentially comprising efficacy. Preliminary data suggest that approximately 25-50% of the animals develop such a response.

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The results of each of the Vitaxin treatments described above is shown in Figure 6B and 6C. In the rabbits receiving treatments on day 1, inhibition of tumor growth was observed in both the 1 mg/kg and the 5 mg/kg dosing groups compared to the control PBS treated control. Specifically, a growth inhibition of about 67 and 80% was observed, respectively, as measured by the mean tumor weight. A lesser degree of inhibition was observed in animals that began Vitaxin treatment on day 7 post implantation. These results are shown in Figure 6C. In all cases, inhibition of tumor growth was not seen at Vitaxin concentrations lower than 0.2 mg/kg.

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EXAMPLE V

Construction of LM609 Grafted Functional Antibody
Fragments

This Example shows the construction of functional LM609 grafted antibody fragments in which only the CDRs have been transferred from the LM609 donor antibody to a human acceptor framework.

CDR grafting of LM609 to produce a functional antibody fragment was accomplished by the methods set forth below. These procedures are applicable for the CDR grafting of essentially any donor antibody where amino acid residues outside of the CDRs from the donor antibody are not desired in the final grafted product.

Briefly, the protein sequence of the LM609 antibody, was determined by cloning and sequencing the cDNA that encodes the variable regions of the heavy and light chains as described in Example I. The CDRs from the LM609 donor antibody were identified and grafted into homologous human variable regions of a human acceptor framework. Identification of CDR regions were based on the combination of definitions published by Kabat et al., and MacCallum et al.

The boundaries of the CDR regions have been cumulatively defined by the above two publications and are residues 30-35, 47-66 and 97-106 for CDRs 1, 2 and 3, respectively, of the heavy chain variable region and residues 24-36, 46-56, and 89-97 for CDRs 1, 2 and 3, respectively, of the light chain variable region. Non-identical donor residues within these boundaries but outside of CDRs as defined by Kabat et al. were identified and were not substituted into the acceptor

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framework. Instead, functional non-donor amino acid residues were identified and substituted for certain of these non-identical residues.

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As described below, the only non-identical residue outside of the CDRs as defined by Kabat et al. but within the CDRs as defined above is at position 49 of the LM609 light chain. To identify functional non-donor amino acids at this position, a library of nineteen antibodies was constructed that contained all non-donor amino acids at position 49 and then screened for binding activity against $\alpha\beta_3$.

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Human immunoglobulin sequences were identified from the Brookhaven Protein Data Bank-Kabat Sequences of Proteins of Immunological Interest database (release 5.0). Human framework sequences showing significant identity to the murine LM609 variable region gene sequences were selected for receiving the LM609 CDRs. Human heavy chain variable region M72 'CL had 88% identity to frameworks 1, 2 and 3 of LM609 heavy chain and human light chain V region LS1 'CL had 79% identity to frameworks 1, 2 and 3 of LM609 light chain. With the exclusion of non-identical residues outside of the CDRs as defined by Kabat et al. murine LM609 CDR sequences as defined by Kabat et al. and MacCallum et al. were grafted onto the human frameworks. Using this grafting scheme, the final grafted product does not contain any amino acid residues outside of the CDRs as defined by Kabat et al. which are identical to an LM609 amino acid at the corresponding position (outside of residues: 31-35, 50-66 and 99-106 for CDRs 1, 2 and 3, respectively, of the heavy chain variable region and residues 24-34, 50-56, and 89-97 for CDRs 1, 2 and 3, respectively, of the light chain variable region). Moreover, no intermediates are

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produced which contain an amino acid residue outside of the CDRs as defined by Kabat et al. which are identical to the LM609 amino acid at that position. The CDR grafting procedures are set forth below.

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Full-length CDR grafted variable region genes were synthesized by PCR using long overlapping oligonucleotides as described previously in Example II. The heavy chain variable region oligonucleotides were those described previously as SEQ ID NOS:9-13. The light chain variable region oligonucleotides were synthesized so as to contain the CDR grafted variable region as well as a stop codon at position 49. The five oligonucleotides for the light chain LM609 grafted variable region are shown as SEQ ID NOS:23-27 where the second oligonucleotide in the series contains the stop codon at position 49 (SEQ ID NO:24). The nucleotide sequences of oligonucleotides used to construct LM609 grafted light chain variable region is shown in Table 7.

Table 7: Oligonucleotides Used to Construct LM609

Grafted Light Chain Variable Region

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GAGATTGTGC	TAACTCAGTC	TCCAGCCACC	CTGTCTCTCA	GCCCAGGAGA	
AAGGGCGACT	CTTCCTGCC	AGGCCAGCCA	AAGTATT	SEQ ID NO: 23	
TTAGATGAGA	AGCCTTGGGG	CTTGACCAGG	CCTTTGTTGA	TACCAGTGTA	
GGTGGTTGCT	AATACTTGG	CTGGC		SEQ ID NO: 24	
CCAAGGCTTC	TCATCTAATA	TCGTTCCCAG	TCCATCTCTG	GGATCCCCGC	
CAGGTTCAGT	GGCAGTGGAT	CAGGGACAGA	TTTC	SEQ ID NO: 25	
GCTGCCACTC	TGTTGACAGT	AATAGACTGC	AAAATCTTCA	GGCTCCAGAC	
TGGAGATAGT	GAGGGTGAAA	TCTGTCCCTG	A	SEQ ID NO: 26	
CAACAGAGTG	GCAGCTGGCC	TCACACGTTC	GGAGGGGGGA	CCAAGGTGGA	
AATTAAG				SEQ ID NO: 27	

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All long oligonucleotides were gel purified. CDR grafting of the LM609 heavy chain variable region was constructed by mixing 5 overlapping oligonucleotides (SEQ ID NOS:9-13), at equimolar concentrations, in the presence of annealing PCR primers containing at least 18 nucleotide residues complementary to vector sequences for the efficient annealing of the amplified V region product to the single-stranded vector. The annealed mixture was fully converted to a double-stranded molecule with T4 DNA polymerase plus dNTPs and ligated with T4 ligase. The mutagenesis reaction (1 μ l) was electroporated into *E. coli* strain DH10B (BRL), titered onto a lawn of XL-1 (Stratagene, Inc.) and incubated until plaques formed. Replica filter lifts were prepared and plaques containing V_H gene sequences were screened either by hybridization with a digoxigenin-labeled oligonucleotide complementary to LM609 heavy chain CDR 2 sequences or reactivity with 7F11-alkaline phosphatase conjugate, a monoclonal antibody raised against the decapeptide sequence Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended to the carboxy terminus of the vector CH_1 domain (Biosite, Inc., San Diego, CA).

Fifty clones that were double-positive were pooled and used to prepare uridynylated template for hybridization mutagenesis with the amplified CDR grafted LM609 V_L product constructed in a similar fashion using the five overlapping oligonucleotides shown as SEQ ID NOS:23-27. The mutagenesis reaction was electroporated into *E. coli* strain DH10B. Randomly picked clones were sequenced to identify a properly constructed template for construction of the non-donor library at position 49. This template was prepared as a uridynylated template and an oligonucleotide population of the following sequence was used for site directed mutagenesis.

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GGGAACGATA-19aa-GATGAGAAGC

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The sequence 19aa in the above primer (SEQ ID NO:30) represents the fact that this primer specifies a sequence population consisting of 19 different codon sequences that encode each of the 19 non-donor amino acids. These amino acids are those not found at position 49 of LM609 and include all amino acids except for Lys. Clones that resulted from this mutagenesis were picked and antibody expressed by these clones were prepared. These samples were then screened for binding to $\alpha\beta$, in an ELISA assay. Clones having either Arg or Met amino acids in position 49 were functionally identified. The nucleotide and amino acid sequence of the LM609 grafted heavy chain variable region is shown in Figure 1A (SEQ ID NOS:1 and 2, respectively). The nucleotide and amino acid sequence of the LM609 grafted light chain variable region is shown in Figure 7 (SEQ ID NOS:31 and 32, respectively).

EXAMPLE VI

Generation of LM609 Grafted Antibodies Having Enhanced Activity

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This example shows *in vitro* maturation of LM609 grafted antibody to obtain antibody variants having increased affinity to $\alpha\beta$, relative to the parent LM609 grafted antibody.

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To optimize the affinity of LM609 grafted antibody *in vitro*, an M13 phage system was used, which permits the efficient synthesis, expression, and screening of libraries of functional antibody fragments (Fabs). The contribution of each of the six CDRs of the Ig heavy and light chains was assessed. The CDRs were

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5 defined broadly based on a combination of sequence
variability and antibody structural models (Kabat et al.,
10 J. Biol. Chem. 252:6609-6616 (1977); Chothia et al.,
supra; MacCallum et al., *supra*). Thus, one library was
5 constructed for each CDR, with the exception of H2 which
was split into two libraries due to its long (20 amino
15 acids) length. The variable region frameworks which
harbored the mutated CDRs were the heavy chain variable
region shown in Figure 1a (SEQ ID NO:2) and the light
10 chain variable region shown in Figure 7 (SEQ ID NO:32).

20 CDRs were chosen from the heavy chain variable
region shown in Figure 1a (SEQ ID NO:2) and the light
chain variable region shown in Figure 7 (SEQ ID NO:32).
Briefly, utilizing the numbering system of Kabat et al.,
25 *supra*, the residues chosen for mutagenesis of the CDRs
(Table 9) were: Gln²⁴-Tyr³⁶ in light chain CDR1 (L1);
Leu⁴⁶-Ser⁵⁶ in light chain CDR2 (L2); Gln⁸⁹-Thr⁹⁷ in light
chain CDR3 (L3); Gly²⁶-Ser³⁵ in heavy chain CDR1 (H1);
30 Trp⁴⁷-Gly⁶⁵ in heavy chain CDR2 (H2); and Ala⁹³-Tyr¹⁰² in
20 heavy chain CDR3 (H3). Libraries were created for each
CDR, with the oligonucleotides designed to mutate a
single CDR residue in each clone. Due to the extended
35 length of H2, two libraries mutating residues 47-55 (H2a)
and 56-65 (H2b), respectively, were constructed to cover
25 this region.

40 The template for generating light chain CDR3
mutants contained Gly at position 92. However, it was
subsequently determined that position 92 of the light
45 chain CDR3 was inadvertently deduced to be a Gly,
30 resulting in humanized LM609 grafted antibodies being
constructed with Gly at that position. It was later
realized that the original LM609 sequence contained an
50 Asn at position 92. Using the methods described herein

5 to introduce mutations into CDRs of an LM609 grafted
antibody, an LM609 grafted antibody having Asn at
position 92 of light chain CDR3 was found to have $\alpha_v\beta_3$
10 binding activity (see Table 9), confirming the
5 identification of Asn⁹² as a functional LM609 grafted
antibody. Thus, antibodies containing light chain CDR3
having Gly or Asn at position 92 are active in binding
15 $\alpha_v\beta_3$.

Oligonucleotides encoding a single mutation
10 were synthesized by introducing NN(G/T) at each CDR
position as described previously (Glaser et al., *supra*).
The antibody libraries were constructed in M131XL604
vector by hybridization mutagenesis as described
25 previously, with some modifications (Rosok et al., J.
15 Biol. Chem. 271:22611-22618 (1996); Huse et al., J.
Immunol. 149:3914-3920 (1992); Kunkel, Proc. Natl. Acad.
Sci. USA 82:488-492 (1985); Kunkel et al., Methods
30 Enzymol. 154:367-382 (1987)). Briefly, the
oligonucleotides were annealed at a 20:1 molar ratio to
20 uridynylated LM609 grafted antibody template (from which
the corresponding CDR had been deleted) by denaturing at
35 85°C for 5 min, ramping to 55°C for 1 h, holding at 55°C
for 5 min, then chilling on ice. The reaction was
extended by polymerization and electroporated into DH10B
25 and titered onto a lawn of XL-1 Blue. The libraries
consisted of pools of variants, each clone containing a
single amino acid alteration in one of the CDR positions.
Utilizing codon-based mutagenesis, every position in all
45 of the CDRs was mutated, one at a time, resulting in the
30 subsequent expression of all twenty amino acids at each
CDR residue (Glaser et al., *supra*). The CDR libraries
ranged in size from 288 (L3) to 416 (L1) unique members
and contained a total of 2336 variants.

5 To permit the efficient screening of the
initial libraries, a highly sensitive plaque lift assay,
termed capture lift, was employed (Watkins et al., Anal.
10 Biochem. 256 (1998)). Briefly, phage expression
5 libraries expressing LM609 grafted antibody variants were
initially screened by a modified plaque lift approach, in
which the nitrocellulose was pre-coated with goat
15 anti-human kappa antibody and blocked with bovine serum
albumin prior to application to the phage-infected
10 bacterial lawn. Following the capture of phage-expressed
LM609 grafted antibody variant Fabs, filters were
20 incubated with 1.0 $\mu\text{g/ml}$ biotinylated $\alpha_v\beta_3$ for 3 h at 4°C,
washed four times, incubated with 2.3 $\mu\text{g/ml}$
NeutrAvidin-alkaline phosphatase (Pierce Chemical Co.;
25 Rockford, IL) for 15 min at 25°C, and washed four times.
All dilutions and washes were in binding buffer.
Variants that bound $\alpha_v\beta_3$ were identified by incubating the
filters for 10-15 min in 0.1 M Tris, pH 9.5, containing
30 0.4 mM 2,2'-di-p-nitrophenyl-5,5'-L-diphenyl-3,
20 3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium
chloride and 0.38 mM 5-bromo-4-chloro-3-indoxyl phosphate
mono-(p-toluidinium) salt (JBL Scientific, Inc.; San Luis
35 Obispo, CA).

To generate biotinylated $\alpha_v\beta_3$, the $\alpha_v\beta_3$ receptor
25 was purified from human placenta by affinity
40 chromatography, as described previously (Smith and
Cheresh, J. Biol. Chem. 263:18726-18731 (1988)). To
biotinylate $\alpha_v\beta_3$, purified receptor was dialyzed into 50
mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mM CaCl_2 , containing
45 0.1% NP-40 (binding buffer) and incubated with 100-fold
30 molar excess sulfosuccinimidobiotin for 3h at 4°C. The
reaction was terminated by the addition of 50 mM
ethanolamine.
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Phage expressed LM609 grafted antibody variants were selectively captured on nitrocellulose filters coated with goat anti-human kappa chain antibody, probed with biotinylated $\alpha_v\beta_3$, and detected with NeutrAvidin-alkaline phosphatase. Initially, biotinylated $\alpha_v\beta_3$ was titrated on lifts containing phage expressing the LM609 grafted antibody parent molecule only. Subsequently, the concentration of biotinylated $\alpha_v\beta_3$ was decreased to yield a barely perceptible signal. In this way, only clones expressing higher affinity variants were readily identified during screening of the variant libraries. Following the exhaustive capture lift screening of >2500 clones from each library, 300 higher affinity variants were identified (see Table 8). The greatest number of clones displaying improved affinity were identified in the H3 (185) and L3 (52) CDRs, though variants with improved affinity were identified in every CDR.

LM609 grafted antibody variants identified by capture lift as having $\alpha_v\beta_3$ binding activity were further characterized to determine binding affinity to $\alpha_v\beta_3$, specificity for $\alpha_v\beta_3$ over other integrins, and $\alpha_v\beta_3$ association and dissociation rates. For these assays, purified Fab of LM609 grafted antibody variants was used. Briefly, Fab was expressed as described previously and was released from the periplasmic space by sonic oscillation (Watkins et al., *supra*, 1997). Cells collected from one liter cultures were lysed in 10 ml 50 mM Tris, pH 8.0, containing 0.05% Tween 20. Fab was bound to a 1 ml protein A column (Pharmacia) which had been equilibrated with 50 mM glycine, pH 8, containing 250 mM NaCl, washed with the same buffer, and eluted with 10 ml of 100 mM glycine, pH 3, into one-tenth volume 1 M

Tris, pH 8. Purified Fab was quantitated as described previously (Watkins et al., *supra*, 1997).

LM609 grafted antibody variants were tested for binding to $\alpha_v\beta_2$ and specificity of binding to $\alpha_v\beta_3$ relative to $\alpha_v\beta_5$ and $\alpha_{IIIb}\beta_3$. For ELISA titration of Fab on immobilized $\alpha_v\beta_3$ and the related integrins $\alpha_v\beta_5$ and $\alpha_{IIIb}\beta_3$, Immulon II microtiter plates were coated with 1 μ g/ml purified receptor in 20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1 mM MnCl_2 , washed once, and blocked in 3% BSA in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 for 1 h at 25°C. Human $\alpha_{IIIb}\beta_3$, purified from platelets, was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and $\alpha_v\beta_5$ was purified from placental extract depleted of $\alpha_v\beta_3$, as described previously (Smith et al., *J. Biol. Chem.* 265:11008-11013 (1990)). Just prior to use, the plates were washed two times and were then incubated 1 h at 25°C with various dilutions of Fab. The plates were washed five times, incubated 1 h at 25°C with goat anti-human kappa-alkaline phosphatase diluted 2000-fold, washed five times, and developed as described previously (Watkins et al., *supra*, 1997). All dilutions and washes were in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM CaCl_2 , and 1 mM MgCl_2 .

Table 8: Capture Lift Screening of LM609 grafted antibody CDR Libraries.

Library	Size ¹	Screened ²	Positives ³	Enhanced Affinity ⁴
H1	320	2500	16	8
H2a	320	5000	26	7
H2b	320	5000	2	1
H3	320	5000	185	78 ⁵
L1	416	2500	12	1
L2	352	3250	7	1
L3	288	5000	52	41

¹Number of unique clones based on DNA sequence. Thirty-two codons are used to express all twenty amino acids at each position.

²Phage-expressed libraries were plated on XL-1 Blue/agar lawns at 500-100 plaques per 100 mm dish.

³Positives are defined as clones that were identified in the initial screen, replated, and verified in a second capture lift assay.

⁴Soluble Fab was titrated against immobilized $\alpha_v\beta_3$ in an ELISA format. Based on comparison of the inflection point of the titration profiles, clones which displayed ~3-fold enhanced affinity were selected for further characterization.

⁵Of the 185 positive clones identified by capture lift, 98 were further characterized for binding to immobilized $\alpha_v\beta_3$.

Figure 8 shows titration of antibody variants and LM609 grafted antibody Fab on immobilized $\alpha_v\beta_3$. Bacterial cell lysates containing LM609 grafted antibody (closed circles), variants with improved affinity isolated from the primary libraries (S102, closed squares; Y100, open squares; and Y101, open triangles) or from the combinatorial libraries (closed triangles), or an irrelevant Fab (open circles) were titrated on immobilized $\alpha_v\beta_3$.

Comparison of the inflection points of the binding profiles obtained from titrating variants on immobilized $\alpha_v\beta_3$ demonstrated that multiple clones displayed >3-fold improved affinity, confirming the effectiveness of utilizing the capture lift in a semi-quantitative fashion (Figure 8, compare squares and open triangles with closed circles). Based on the capture lift screening and subsequent characterization of binding to immobilized $\alpha_v\beta_3$, it was concluded that both heavy and light chain CDRs are directly involved in the interaction of $\alpha_v\beta_3$ with the LM609 grafted antibody variants.

DNA was isolated from clones displaying >3-fold enhanced binding and sequenced to identify the mutations which resulted in higher affinity. DNA sequencing was performed on isolated single-stranded DNA. The heavy and light chain variable region genes were sequenced by the fluorescent dideoxynucleotide termination method (Perkin-Elmer; Foster City, CA). Based on sequence analysis of 103 variants, 23 unique mutations clustered at 14 sites were identified (Table 9). The majority of the sites of beneficial mutations were found in the heavy chain CDRs, with four located in H3, and three each in H2 (2a and 2b combined) and H1. Seven distinct and

beneficial amino acid substitutions were identified at a single site within H3, tyrosine residue 102. The diverse nature of the substitutions at this site suggests that tyrosine residue 102 may sterically hinder LM609 grafted antibody binding to $\alpha_v\beta_3$. In support of this, variants expressing the other aromatic amino acids (phenylalanine, histidine, and tryptophan) instead of tyrosine at residue 102 were never isolated following screening for enhanced binding.

The affinities of select variants were further characterized by utilizing surface plasmon resonance (BIAcore) to measure the association and dissociation rates of purified Fab with immobilized $\alpha_v\beta_3$. Briefly, surface plasmon resonance (BIAcore; Pharmacia) was used to determine the kinetic constants for the interaction between $\alpha_v\beta_3$ and LM609 grafted antibody variants. Purified $\alpha_v\beta_3$ receptor was immobilized to a (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride)/N-hydroxysuccinimide-activated sensor chip by injecting 30 μ l of 15 μ g/ml $\alpha_v\beta_3$ in 10 mM sodium acetate, pH 4. To obtain association rate constants (k_{on}), the binding rate at five different Fab concentrations, ranging from 5-40 μ g/ml in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM CaCl₂, and 1 mM MgCl₂, was determined at a flow rate of 10 μ l/min. Dissociation rate constants (k_{off}) were the average of five measurements obtained by analyzing the dissociation phase at an increased flow rate (40 μ l/min). Sensorgrams were analyzed with the BIAevaluation 2.1 program (Pharmacia). Residual Fab was removed after each measurement with 10 mM HCl, 2 mM CaCl₂ and 1 mM MgCl₂.

5 Table 9 shows that the variants all displayed a lower K_d than the LM609 grafted antibody parent molecule, consistent with both the capture lift and the ELISA.

10 Analysis of association and dissociation rates revealed

5 that the majority of improved variants had slower dissociation rates while having similar association rates. For example, LM609 grafted antibody had an association rate $18.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, while the variants ranged from $16.7\text{--}31.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. In contrast, every
10 clone dissociated slower than LM609 grafted antibody ($4.97 \times 10^{-3} \text{ s}^{-1}$) with dissociation rates ranging from
20 1.6-fold ($3.03 \times 10^{-3} \text{ s}^{-1}$) to 11.8-fold ($0.42 \times 10^{-3} \text{ s}^{-1}$) slower.

25 These results demonstrate that introducing
15 single amino acid substitutions into LM609 grafted antibody CDRs allows the identification of modified LM609 grafted antibodies having higher affinity for $\alpha_v\beta_3$ than
30 the parent LM609 grafted antibody.

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Table 9: Identification of Enhanced LM609 Grafted Antibodies from Primary Libraries

chain	library†	sequence	k_{on} ($\times 10^4$) ($M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$) (s^{-1})	K_d (nM)
	LM609 grafted antibody		18.0	4.97	27.6
H	CDR1 T27 W29 L30	G F T F S S Y D M S W L	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.
H	CDR2a K52	W V A K V S S G G G K	17.8	2.18	12.2
H	CDR2b P60 E64	S T Y Y L D T V Q G P E	31.8 n.d.	1.85 n.d.	5.8 n.d.
H	CDR3 H97 Y100 D101 Y101 S102 T102 D102 E102 M102 G102 A102	A R H N Y G S F A Y H Y D Y S T D E M G A	22.0 17.5 n.d. 21.8 24.2 24.6 27.6 n.d. n.d. 16.1 27.5	3.03 2.51 n.d. 0.48 1.44 1.43 0.97 n.d. n.d. 2.01 2.27	13.8 14.3 n.d. 2.2 6.0 5.8 3.5 n.d. n.d. 12.5 8.3
L	CDR1 F32	Q A S Q S I S N H L R W Y F	16.7	0.42	2.5
L	CDR2 S51	L L I R Y R S Q S I S S	n.d.	n.d.	n.d.
L	CDR3 N92 T92 L96 Q96	Q Q S G S W P H T N T L Q	23.6 n.d. 24.3 n.d.	1.35 n.d. 2.23 n.d.	5.7 n.d. 9.2 n.d.

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EXAMPLE VIIGeneration of High Affinity LM609 Grafted Antibodies

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This example shows that single amino acid mutations in CDRs of an LM609 grafted that result in higher affinity binding to $\alpha_v\beta_3$ can be combined to generate high affinity LM609 grafted antibodies.

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Random combination of all of the beneficial mutations of LM609 grafted antibody would generate a combinatorial library containing $>10^5$ variants, requiring efficient screening methodologies. Therefore, to determine if clones displaying >10 -fold enhanced affinities could be rapidly distinguished from one another, variants displaying 3 to 13-fold enhanced affinity were evaluated by capture lift utilizing lower concentrations of biotinylated $\alpha_v\beta_3$. Despite repeated attempts with a broad range of concentrations of $\alpha_v\beta_3$, consistent differences in the capture lift signals were not observed. Because of this, smaller combinatorial libraries were constructed and subsequently screened by ELISA.

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Four distinct combinatorial libraries were constructed in order to evaluate the optimal number of combinations that could be accomplished utilizing two site hybridization mutagenesis (Figure 9). Briefly, combinatorial libraries were constructed by synthesizing degenerate oligonucleotides encoding both the wild-type and beneficial heavy chain mutations (H2, Leu⁶⁰-Pro; H3 Tyr⁹⁷-His; H3, Ala¹⁰¹-Tyr; H3, Tyr¹⁰²-Ser, Thr, Asp, Glu, Met, Gly, Ala). Utilizing two site hybridization mutagenesis, as described above, the oligonucleotides were annealed at a 40:1 molar ratio to uridynylated template prepared from LM609 grafted antibody and three

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light chain mutations (Figure 9; L1, His³²-Phe; L3, Gly⁹²-Asn; L3, His⁹⁶-Leu). As a result, a total of 256 variants were synthesized in four combinatorial library subsets.

Figure 9 shows construction of combinatorial libraries of beneficial mutations. Uridinylated template from LM609 grafted antibody and three optimal light chain variants (F32, N92, and L96) was prepared. Two site hybridization was performed with two degenerate oligonucleotides, which were designed to introduce beneficial mutations at four distinct heavy chain residues.

Following preparation of uridinylated templates of LM609 grafted antibody and three light chain variants, (Table 9; F32, N92, and L96), degenerate oligonucleotides encoding the wild type residue and the most beneficial heavy chain mutations (Table 9; P60, H97, Y101, S102, T102, D102, E102, M102, G102, and A102) were hybridized to the light chain templates, resulting in four combinatorial libraries, each containing 64 unique variants. Potentially, the combination of multiple mutations can have detrimental effects on affinity and, thus, can prevent the identification of beneficial combinations resulting from mutations at fewer sites. For this reason, the amino acid expressed by the LM609 grafted antibody parent molecule was included at each position in the combinatorial library. By utilizing this approach, simultaneous combinatorial mutagenesis of three CDRs (L1 or L3 each in combination with H2 and H3) was accomplished. Based on sequence analysis, the two site hybridization mutagenesis was achieved with ~50% efficiency.

5 In order to screen the combinatorial libraries,
soluble Fab was expressed and released from the periplasm
of small-scale (<1 ml) bacterial cultures that had been
10 infected with randomly selected clones. Although
5 variable expression levels were observed, uniform
quantities of the unpurified variants were captured on a
microtiter plate through a peptide tag present on the
15 carboxyl-terminus of the heavy chain. Briefly,
combinatorial LM609 grafted antibody libraries were
10 screened by an ELISA that permits the determination of
relative affinities of antibody variants produced in
20 small-scale bacterial cultures (Watkins et al., Anal.
Biochem. 253:37-45 (1997)). An Immulon II microtiter
plate (Dynatech Laboratories; Chantilly, VA) was coated
15 with 10 µg/ml of the 7F11 monoclonal antibody, which
25 recognizes a peptide tag on the carboxyl-terminus of the
LM609 grafted antibody variant heavy chains (Field et
al., Mol. Cell. Biol. 8:2159-2165 (1988)). Following
30 capture of Fab from *E. coli* lysates, the plate was
20 incubated with 0.5-1 µg/ml biotinylated $\alpha_v\beta_3$ for 1 h at
25°C. The plate was washed seven times, incubated with
0.5 U/ml streptavidin-alkaline phosphatase (1000 U/ml;
35 Boehringer Mannheim; Indianapolis, IN) for 15 min at
25°C, washed seven times, and developed as described
25 previously (Watkins et al., *supra*, 1997). All dilutions
and washes were in binding buffer.

40 As described previously (Watkins et al., *supra*,
1997), this ELISA screening method enabled a rapid and
direct comparison of the relative affinities of the
45 variants following incubation with biotinylated $\alpha_v\beta_3$ and
30 streptavidin-alkaline phosphatase. To ensure that the
full Fab diversity was sampled, one thousand randomly
selected clones were screened from each combinatorial
50 library. Variants that displayed an enhanced ELISA

5 signal were further characterized for binding to
immobilized $\alpha_v\beta_3$ (Figure 8, closed triangles) and were
sequenced to identify the mutations (Table 10).

10 Screening of the four combinatorial libraries
5 identified fourteen unique combinations of mutations that
improved binding significantly over the individual
15 mutations identified in the screening of the first
library. While the best clone from the primary screen
had a 12.5-fold increase in affinity, the fourteen unique
20 combinations isolated from screening the combinatorial
libraries displayed affinities ranging from 18 to 92-fold
greater than the parent LM609 grafted antibody. The
majority of these variants consisted of H2 and H3
25 mutations combined with the L1 or L3 mutations.
15 Beneficial combinations of heavy chain mutations with
wild-type light chain were also identified, but did not
result in improved affinity to the same extent as other
combinatorial variants. The variants predominantly
30 contained 2 to 4 mutations, with one clone, C29,
20 containing five mutations. No direct correlation between
the total number of mutations in each variant and the
35 resulting affinity was observed. For example, while the
binding of clone C37 was 92-fold enhanced over the parent
molecule and was achieved through the combination of
25 three mutations, clone C29 had ~55-fold greater affinity
40 achieved through the combination of five mutations.
Multiple variants displaying >50-fold enhanced affinity
resulting from the combination of as few as two mutations
were identified (2G4, 17, and V357D).

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30 The combinatorial clones with improved affinity
all displayed >10-fold slower dissociation rates,
possibly reflecting a selection bias introduced by long
50 incubation steps in the screening. In addition, all of

5 the combinatorial variants isolated from the library
based on the L96 light chain mutation also displayed 2 to
4-fold greater association rates. Previously, it has
10 been demonstrated that the antibody repertoire shifts
5 towards immunoglobulins displaying higher association
rates during affinity maturation *in vivo* (Foote and
Milstein, Nature 352:530-532 (1991)). The L96 subset of
15 variants, therefore, may more closely mimic the *in vivo*
affinity maturation process where B-lymphocyte
10 proliferation is subject to a kinetic selection.

20 LM609 grafted antibody binds the $\alpha_v\beta_3$ complex
specifically and does not recognize either the α_v or the
 β_3 chain separately. To further characterize the
variants, clones were screened for reactivity with the
25 related integrins, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_5$. All variants tested
15 were unreactive with both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_5$, consistent with
the improved binding not substantially altering the
interaction of Fab and receptor.
30

As a first step toward determining if the
20 increase in affinity of the variants resulted in greater
biological activity, variants displaying a range of
35 affinities were assayed for their ability to inhibit the
binding of a natural ligand, fibrinogen, to immobilized
 $\alpha_v\beta_3$ receptor. Briefly, LM609 grafted antibody variants
40 25 were tested for inhibition of ligand binding as described
previously except that the binding of biotinylated human
fibrinogen (Calbiochem, La Jolla, CA) was detected with
0.5 μ g/ml NeutrAvidin-alkaline phosphatase (Smith et al.,
45 J. Biol. Chem. 265:12267-12271 (1990)).

Table 10: Identification of Optimal Combinatorial Mutations

library*	clone	L1 32	L3 92	L3 96	H2 60	H3 97	H3 101	H3 102	k_{on} ($\times 10^4$) ($M^{-1}s^{-1}$)	k_{off} ($\times 10^{-3}$) (s^{-1})	Kd (nM)
wild type		H	G	H	L	Y	A	Y	18.0	4.97	27.6
F32	17	F						S	25.1	0.138	0.5
	7	F			P	H		S	20.4	0.236	1.2
	56	F			P			S	26.6	0.135	0.5
	C59	F			P			D	26.5	0.137	0.5
	C176	F			P			T	22.5	0.192	0.9
	V357D	F						D	27.9	0.140	0.5
N92	C119		N		P			S	21.5	0.316	1.5
L96	8F9			L	P	H		S	47.5	0.280	0.6
	C29			L	P	H	Y	S	67.5	0.343	0.5
	2G4			L				S	60.3	0.229	0.4
	6H6			L		H		S	50.4	0.187	0.4
	C37			L			Y	E	44.8	0.147	0.3
	6D1			L	P		Y	S	41.0	0.158	0.4
	6G1			L	P			S	38.9	0.280	0.7

5 The results of these competition assays are
shown in Figure 10. Figure 10A shows inhibition of
fibrinogen binding to immobilized $\alpha_v\beta_3$. Immobilized $\alpha_v\beta_3$
10 was incubated with 0.1 $\mu\text{g/ml}$ biotinylated fibrinogen and
5 various concentrations of LM609 grafted antibody (open
circles), S102 (closed circles), F32 (open triangles), or
C59 (closed triangles) for 3 h at 37°C. Unbound ligand
15 and Fab were removed by washing and bound fibrinogen was
quantitated following incubation with NeutrAvidin
10 alkaline phosphatase conjugate. Figure 10B shows
correlation of affinity of variants with inhibition of
20 fibrinogen binding. The concentration of variants
required to inhibit the binding of fibrinogen to
immobilized $\alpha_v\beta_3$ by 50% (IC_{50}) was plotted as a function of
25 the affinity (Kd).

As shown in Figure 10A, higher affinity
variants were more effective at blocking the ligand
30 binding site of the receptor (compare LM609 grafted
antibody, open circles, with any of the variants).
20 Subsequent analysis of ten variants displaying affinities
(Kd) ranging from 0.3 to 27 nm demonstrated a good
35 correlation ($r^2 = 0.976$) between affinity and ability to
inhibit fibrinogen binding (Figure 10B). In addition,
the variants were tested for inhibition of vitronectin
25 binding to the receptor. Similar to fibrinogen, the
40 variants were more effective at inhibiting the
interaction than the parent molecule. Thus, consistent
with the cross-reactivity studies with related integrin
receptors, mutations which increased affinity did not
45 appear to substantially alter the manner in which the
antibody interacted with the receptor.

5 The ability of the variants to inhibit the
adhesion of M21 human melanoma cells expressing the $\alpha_v\beta_3$
receptor to fibrinogen was examined. Inhibition of the
10 adhesion of 4×10^4 M21 cells to fibrinogen by the LM609
5 grafted antibody variants was performed as described
previously (Leavesley et al., J. Cell Biol. 117:1101-1107
(1992)). Similar to the ligand competition studies with
15 purified fibrinogen and $\alpha_v\beta_3$ receptor, higher affinity
variants were generally more effective at preventing cell
10 adhesion than was LM609 grafted antibody (Figure 11).
Figure 11 shows inhibition of M21 human melanoma cell
20 adhesion to fibrinogen. Cells and various concentrations
of LM609 grafted antibody Fab (closed triangles), S102
(open circles), G102 (closed circles), or C37 (open
15 triangles) were added to 96 well cell culture plates
25 which had been coated with 10 $\mu\text{g/ml}$ fibrinogen. After
incubating for 35 min at 37°C, unbound cells were removed
by washing and adherent cells were quantitated by crystal
30 violet staining.

20 Although intact LM609 grafted antibody Ig
inhibits cell adhesion, the phage expressed Fab did not
35 affect cell adhesion at concentrations as high as 1 mg/ml
(Figure 11, closed triangles). Clone C37, isolated from
the combinatorial library and displaying ~90-fold greater
25 affinity than LM609 grafted antibody Fab, inhibited cell
adhesion completely (Figure 11, open triangles). Variant
40 G102 had a moderately higher affinity (2.2-fold enhanced)
and also inhibited cell adhesion, though less effectively
than C37 (Figure 11, closed circles). Surprisingly,
45 clone S102 (Figure 11, open circles), which had a
30 4.6-fold higher affinity than LM609 grafted antibody, was
ineffective at inhibiting cell adhesion, suggesting that
clones G102 and S102 interact with the $\alpha_v\beta_3$ receptor
50 differently.

5 These results show that combining single amino
acid mutations that result in LM609 grafted antibodies
exhibiting higher binding affinity to $\alpha_v\beta_3$, allows the
10 identification of high affinity LM609 grafted antibody
5 mutants having greater than 90-fold higher binding
affinity than the parent LM609 grafted antibody.

15 EXAMPLE VIII

Generation of High Affinity Enhanced LM609 Grafted Antibodies

20 10 This example describes the generation of high
affinity enhanced LM609 grafted antibodies having
increased stability.

25 The high affinity clone 6H6 (see Table 10 in
Example VII) was further characterized and was found to
15 exhibit some proteolysis. Therefore, to identify
30 variants having increased stability, 32 codon variants
were introduced simultaneously at each of positions Asn⁹⁶
and His⁹⁷ (numbering according to Kabat et al., *supra*) in
the heavy chain CDR3 of 6H6 (SEQ ID NO:94), and the
35 20 variants were screened for $\alpha_v\beta_3$ binding activity. Those
variants that retained binding activity were sequenced
and then screened for susceptibility to proteolysis. The
40 variant 6H6LH was identified as exhibiting $\alpha_v\beta_3$ binding
25 activity and resistance to proteolysis (see Tables 11 and
15). The 6H6LH variant, which had high affinity $\alpha_v\beta_3$
binding activity, had Leu substituted for Asn⁹⁶ but
retained His⁹⁷.

45 Although the 6H6LH variant exhibited high
affinity binding to $\alpha_v\beta_3$, the affinity was somewhat lower
50 30 than the 6H6 variant (see Table 15). Therefore, CDR

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mutations that had been found to give high affinity binding were combined with the 6H6LH variant.

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Specifically, His³² in the light chain CDR1 of 6H6 was mutated to Phe, as in clone F32 (see Table 9), to

5 generate clone 2236/6H6LH (see Table 13). In an additional variant, Leu⁶¹ in the heavy chain CDR2 of 6H6 was also mutated to Pro, as in clone P60 (see Table 9),
15 to generate clone 2236-38/6H6LH (see Table 11).

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Shown in Tables 11 and 12 are amino acid and
10 nucleotide sequences, respectively, of heavy chain CDRs of the enhanced high affinity variants of 6H6. Shown in
Tables 13 and 14 are amino acid and nucleotide sequences, respectively, of light chain CDRs of the enhanced high
affinity variants of 6H6. The CDRs shown are according
25 to Chothia, *supra*. The amino acids and codons that
15 differ from the 6H6 variant are shown in bold.

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Table 11: Amino Acid Sequences of Heavy Chain CDRs of
High Affinity Enhanced 6H6 Variant Antibodies

Heavy Chain CDR1

Clone

5	6H6LH	G F T F S S Y D M S	SEQ ID NO:34
	2236/6H6LH	G F T F S S Y D M S	SEQ ID NO:34
	2236-38/6H6LH	G F T F S S Y D M S	SEQ ID NO:34

Heavy Chain CDR2

Clone

10	6H6LH	K V S S G G G S T Y	SEQ ID NO:102
		Y L D T V Q G	
	2236/6H6LH	K V S S G G G S T Y	SEQ ID NO:102
		Y L D T V Q G	
	2236-38/6H6LH	K V S S G G G S T Y	SEQ ID NO:104
		Y P D T V Q G	

Heavy Chain CDR3

	6H6LH	H L H G S F A S	SEQ ID NO:106
	2236/6H6LH	H L H G S F A S	SEQ ID NO:106
	2236-38/6H6LH	H L H G S F A S	SEQ ID NO:106

**Table 12: Nucleotide Sequences of Heavy Chain CDRs of
High Affinity Enhanced 6H6 Variant Antibodies**

Heavy Chain CDR1	
6H6LH	GGA TTC ACC TTC AGT AGC TAT GAC
5	ATG TCT SEQ ID NO:33
2236/6H6LH	GGA TTC ACC TTC AGT AGC TAT GAC
15	ATG TCT SEQ ID NO:33
2236-38/6H6	GGA TTC ACC TTC AGT AGC TAT GAC
	ATG TCT SEQ ID NO:33
Heavy Chain CDR2	
6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC
	ACC TAC TAT TTA GAC ACT GTG CAG
	GGC SEQ ID NO:101
2236/6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC
15	ACC TAC TAT TTA GAC ACT GTG CAG
	GGC SEQ ID NO:101
2236-38/6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC
30	ACC TAC TAT CCA GAC ACT GTG CAG
	GGC SEQ ID NO:103
Heavy Chain CDR3	
6H6LH	CAT CTT CAT GGC AGT TTT GCT TCT
	SEQ ID NO:105
2236/6H6LH	CAT CTT CAT GGC AGT TTT GCT TCT
40	SEQ ID NO:105
2236-38/6H6LH	CAT CTT CAT GGC AGT TTT GCT TCT
25	SEQ ID NO:105

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**Table 13: Amino Acid Sequences of Light Chain CDRs of
High Affinity Enhanced 6H6 Variant Antibodies**

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Light Chain CDR1

6H6LH	Q A S Q S I S N H L H	SEQ ID NO:108
5 2236/6H6LH	Q A S Q S I S N F L H	SEQ ID NO:110
2236-38/6H6LH	Q A S Q S I S N F L H	SEQ ID NO:110

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Light Chain CDR2

6H6LH	Y R S Q S I S	SEQ ID NO:112
2236/6H6LH	Y R S Q S I S	SEQ ID NO:112
10 2236-38/6H6LH	Y R S Q S I S	SEQ ID NO:112

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Light Chain CDR3

6H6LH	Q Q S G S W P L T	SEQ ID NO:90
2236/6H6LH	Q Q S G S W P L T	SEQ ID NO:90
2236-38/6H6LH	Q Q S G S W P L T	SEQ ID NO:90

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Table 14: Nucleotide Sequences of Light Chain CDRs of High Affinity Enhanced 6H6 Variant Antibodies

Light Chain CDR1	
6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
5	CAC CTA CAC SEQ ID NO:107
2236/6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
15	TTC CTA CAC SEQ ID NO:109
2236-38/6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
	TTC CTA CAC SEQ ID NO:109
Light Chain CDR2	
6H6LH	TAT CGT TCC CAG TCC ATC TCT
	SEQ ID NO:111
2236/6H6LH	TAT CGT TCC CAG TCC ATC TCT
25	SEQ ID NO:111
15	2236-38/6H6LH TAT CGT TCC CAG TCC ATC TCT
	SEQ ID NO:111
Light Chain CDR3	
6H6LH	CAA CAG AGT GGC AGC TGG CCT CTG
	ACG SEQ ID NO:89
20	2236/6H6LH CAA CAG AGT GGC AGC TGG CCT CTG
35	ACG SEQ ID NO:89
2236-38/6H6LH	CAA CAG AGT GGC AGC TGG CCT CTG
	ACG SEQ ID NO:89

The kinetics of $\alpha_v\beta_3$ binding was determined for each clone using surface plasmon resonance (BIAcore) essentially as described in Example VI. The binding kinetics and affinities of the clones are shown in Table 15.

Table 15. Binding Kinetics of Enhanced LM609 Grafted Antibody Clones

Clone ID#	k_{on}	k_{off}	K_d
6H6	2.59E+05	1.60E-04	0.62 nM
2236/6H6LH	2.25E+05	1.48E-04	0.66 nM
6H6LH	3.01E+05	5.48E-04	1.82 nM
2236-38/6H6LH	2.76E+05	2.49E-04	0.90 nM

As shown in Table 15, clones 6H6LH, 2236/6H6LH, and 2236-38/6H6LH had affinities similar to 6H6. The affinity for 6H6 in these experiments was 0.62 nM, compared to 0.4 nM observed in the previous experiment (see Table 10 in Example VII). This difference is due to the observed k_{on} , which is within the variability of the experiments. The on rates were very similar between 6H6, 6H6LH, 2236/6H6LH, and 2236-38/6H6LH. The small difference in the observed K_d appeared to be due primarily to differences in k_{off} .

These results describe additional enhanced LM609 grafted antibodies exhibiting high affinity for $\alpha_v\beta_3$ and resistance to proteolysis.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

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Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

Claims

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What is claimed is:

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1. An enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising a CDR selected from the group consisting of a V_H CDR2 referenced as SEQ ID NO:104; a V_H CDR3 referenced as SEQ ID NO:106; and a V_L CDR1 referenced as SEQ ID NO:110.

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2. The enhanced LM609 grafted antibody of claim 1, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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3. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 1.

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4. An enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising the V_H CDR1 referenced as SEQ ID NO:34; the V_H CDR2 referenced as SEQ ID NO:102; the V_H CDR3 referenced as SEQ ID NO:106; the V_L CDR1 referenced as SEQ ID NO:108; the V_L CDR2 referenced as SEQ ID NO:112; and the V_L CDR3 referenced as SEQ ID NO:90.

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5. The enhanced LM609 grafted antibody of claim 4, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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6. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 4.

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7. An enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising the V_H CDR1 referenced as SEQ ID NO:34; the V_H CDR2 referenced as SEQ ID NO:102; the V_H CDR3 referenced as SEQ ID NO:106; the V_L CDR1 referenced as SEQ ID NO:110; the V_L CDR2 referenced as SEQ ID NO:112; and the V_L CDR3 referenced as SEQ ID NO:90.

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8. The enhanced LM609 grafted antibody of claim 7, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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9. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 7.

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10. An enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising the V_H CDR1 referenced as SEQ ID NO:34; the V_H CDR2 referenced as SEQ ID NO:104; the V_H CDR3 referenced as SEQ ID NO:106; the V_L CDR1 referenced as SEQ ID NO:110; the V_L CDR2 referenced as SEQ ID NO:112; and the V_L CDR3 referenced as SEQ ID NO:90.

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11. The enhanced LM609 grafted antibody of claim 10, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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12. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 10.

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13. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 1.

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14. The nucleic acid molecule of claim 13, wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:103, SEQ ID NO:105, and SEQ ID NO:109.

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15. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 4.

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16. The nucleic acid molecule of claim 15, wherein said nucleic acid molecule comprises the nucleotide sequence referenced as SEQ ID NO:33 encoding a V_H CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the nucleotide sequence referenced as SEQ ID NO:107 encoding a V_L CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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17. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 7.

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18. The nucleic acid molecule of claim 17, wherein said nucleic acid molecule comprises the nucleotide sequence referenced as SEQ ID NO:33 encoding a V_H CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the nucleotide sequence referenced as SEQ ID NO:109 encoding a V_L CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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19. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 10.

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20. The nucleic acid molecule of claim 19, wherein said nucleic acid molecule comprises the
5 nucleotide sequence referenced as SEQ ID NO:33 encoding a V_H CDR1; the nucleotide sequence referenced as SEQ ID
15 NO:103 encoding a V_H CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V_H CDR3; the
nucleotide sequence referenced as SEQ ID NO:109 encoding
10 a V_L CDR1; the nucleotide sequence referenced as SEQ ID
20 NO:111 encoding a V_L CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V_L CDR3.

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21. A method of inhibiting a function of $\alpha_v\beta_3$, comprising contacting $\alpha_v\beta_3$ with the enhanced LM609 grafted
15 antibody of claim 1.

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22. A method of inhibiting a function of $\alpha_v\beta_3$, comprising contacting $\alpha_v\beta_3$ with the enhanced LM609 grafted
antibody of claim 4.

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23. A method of inhibiting a function of $\alpha_v\beta_3$, comprising contacting $\alpha_v\beta_3$ with the enhanced LM609 grafted
20 antibody of claim 7.

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24. A method of inhibiting a function of $\alpha_v\beta_3$, comprising contacting $\alpha_v\beta_3$ with the enhanced LM609 grafted
antibody of claim 10.

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25. An antibody, or a functional fragment thereof, comprising a CDR selected from the group consisting of a V_H CDR2 referenced as SEQ ID NO:104; a V_H CDR3 referenced as SEQ ID NO:106; and a V_L CDR1 referenced as SEQ ID NO:110, and exhibiting enhanced binding affinity to $\alpha_v\beta_3$, compared to LM609.

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26. The antibody of claim 25, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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27. An antibody, or functional fragment thereof, comprising the V_H CDR1 referenced as SEQ ID NO:34; a V_H CDR2 referenced as SEQ ID NO:102; a V_H CDR3 referenced as SEQ ID NO:106; a V_L CDR1 referenced as SEQ ID NO:108; a V_L CDR2 referenced as SEQ ID NO:112; and a V_L CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced binding activity to $\alpha_v\beta_3$, compared to LM609.

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28. The antibody of claim 27, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

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29. An antibody, or a functional fragment thereof, comprising a V_H CDR1 referenced as SEQ ID NO:34; a V_H CDR2 referenced as SEQ ID NO:102; a V_H CDR3 referenced as SEQ ID NO:106; a V_L CDR1 referenced as SEQ ID NO:110; a V_L CDR2 referenced as SEQ ID NO:112; and a V_L CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced binding activity to $\alpha_v\beta_3$, compared to LM609.

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30. The enhanced LM609 grafted antibody of claim 29, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)₂ and scFV.

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31. An antibody, or a functional fragment thereof, comprising a V_H CDR1 referenced as SEQ ID NO:34; a V_H CDR2 referenced as SEQ ID NO:104; a V_H CDR3 referenced as SEQ ID NO:106; a V_L CDR1 referenced as SEQ ID NO:110; a V_L CDR2 referenced as SEQ ID NO:112; and a V_L CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced binding activity to $\alpha_v\beta_3$ compared to LM609.

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32. The enhanced LM609 grafted antibody of claim 30, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)₂ and scFV.

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33. A nucleic acid molecule having a nucleotide sequence selected from the group of nucleotide sequences consisting of SEQ ID NO:33, SEQ ID NO:89, SEQ ID NO:101; SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:107, SEQ ID NO:109, and SEQ ID NO:111.

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CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTT GTG CAG CCT GGA AGG 48
 Gln Val Gln Leu Val 5 Gly Val 10 Gly Val 15
 TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT 96
 Ser Leu Arg 20 Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Tyr 30
 GAC ATG TCT TGG GTT CGC CAG GCT CCG GGC AAG GGT CTG GAG TGG GTC 144
 Asp Met Ser Trp Val Arg Gln Ala 40 Pro Gly Lys Gly Leu Glu Trp Val 45
 GCA AAA GTT AGT AGT GGT GGT GGT AGC ACC TAC TAT TTA GAC ACT GTG 192
 Ala Lys Val Ser Ser Gly 55 Gly Ser Thr Tyr Tyr Leu Asp Thr Val 60
 CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT AGT AAG AAC ACC CTA TAC 240
 Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr 75 80
 CTG CAA ATG AAC TCT CTG AGA GCC GAG GAC ACA GCC GTG TAT TAC TGT 288
 Leu Gln Met Asn Ser 85 Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys 95
 GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT ACA 336
 Ala Arg His 100 Tyr Gly Ser Phe 105 Tyr Trp Gly Gln Gly Thr 110
 GTG ACT GTT TCT AGT 351
 Val Thr 115

FIG. 1A

GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA	48
Glu Ile Val Leu Thr 5	
GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC	96
Glu Arg Ala Thr Leu 20	
CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC	144
Leu His Trp Tyr Gln Gln Arg Pro Gly Ala Pro Arg Leu Ile	
AAG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC	192
Lys Tyr Arg Ser Gln Ser 55	
AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT	240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr 75	
GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGT GGC AGC TGG CCT CAC	288
Glu Asp Phe Ala Val 85	
ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG	321
Thr Phe Gly Gly Gly Val 105	

FIG. IB

GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA AGG	48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Arg	15
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC GCT TTC AGT AGC TAT	96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr	30
GAC ATG TCT TGG GTT CGC CAG ATT CCG GAG AAG AGG CTG GAG TGG GTC	144
Asp Met Ser Trp Val Arg Gln Ile Pro Glu Lys Arg Leu Glu Trp Val	45
GCA AAA GTT AGT AGT GGT GGT GGT AGC ACC TAC TAT TTA GAC ACT GTG	192
Ala Lys Val Ser Ser Gly Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val	60
CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTA TAC	240
Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr	80
CTG CAA ATG AGC AGT CTG AAC TCT GAG GAC ACA GCC ATG TAT TAC TGT	288
Leu Gln Met Ser Ser Leu Asn Ser Glu Asp Thr Ala Met Tyr Tyr Cys	95
GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT CTG	336
Ala Arg His Asn Tyr Gly Ser Phe Ala Tyr Trp Gly Gln Gly Thr Leu	110
GTC ACT GTC TCT GCA	351
Val Thr Val Ser Ala	115

FIG. 2A

GAT ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT GTG ACA CCA GGA	48
Asp Ile Val Leu Thr 5	
GAT AGC GTC AGT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC	96
Asp Ser Val Ser Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn His	
CTA CAC IGG TAT CAA CAA AAA TCA CAT GAG TCT CCA AGG CTT CTC ATC	144
Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile	
AAG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC TCC AGG TTC AGT GGC	192
Lys Tyr Arg Ser Gln Ser Ile Ser Ile Pro Ser Arg Phe Ser Gly	
AGT GGA TCA GGG ACA GAT TTC GCT CTC AGT ATC AAC AGT GTG GAG ACT	240
Ser Gly Ser Gly Thr Asp Phe Ala Leu Ser Ile Asn Ser Val Glu Thr	
GAA GAT TTT GGA ATG TAT TTC TGT CAA CAG AGT GGC AGC TGG CCT CAC	288
Glu Asp Phe Gly Met Tyr Phe Cys Gln Gln Ser Gly Ser Trp Pro His	
ACG TTC GGA GGG GGG ACC AAG CTG GAA ATT AAG	321
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys	

FIG. 2B

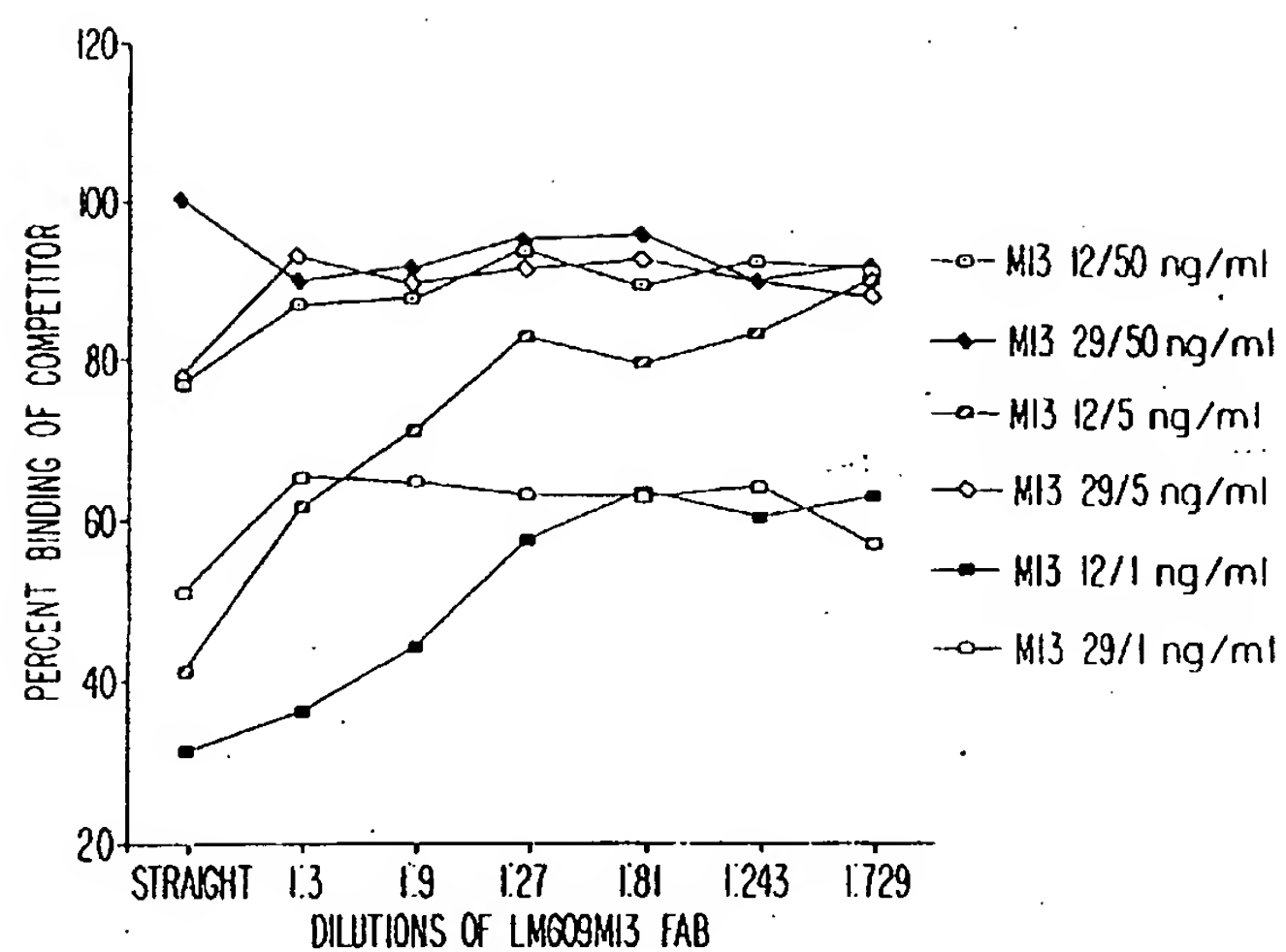


FIG. 3

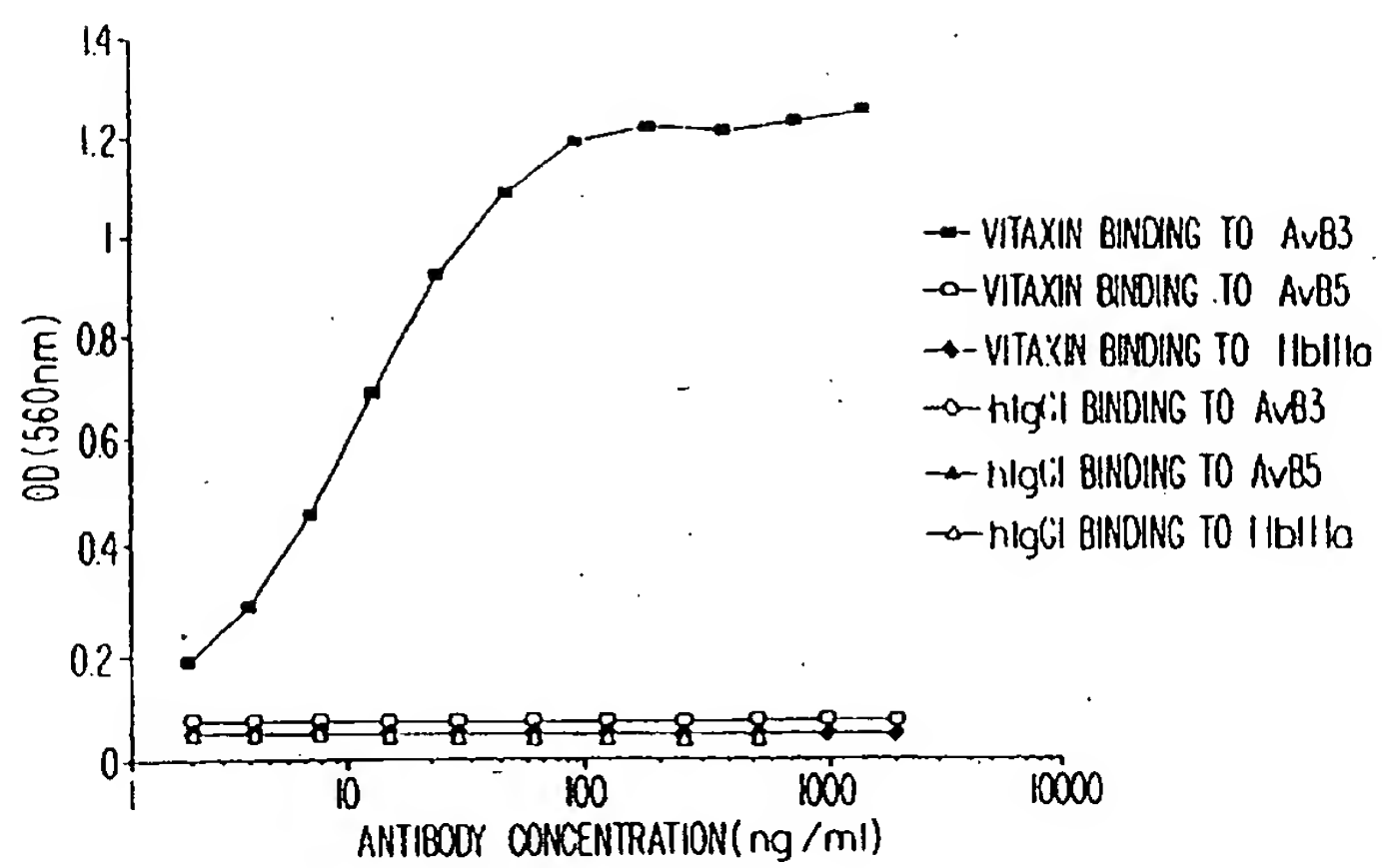


FIG. 4A

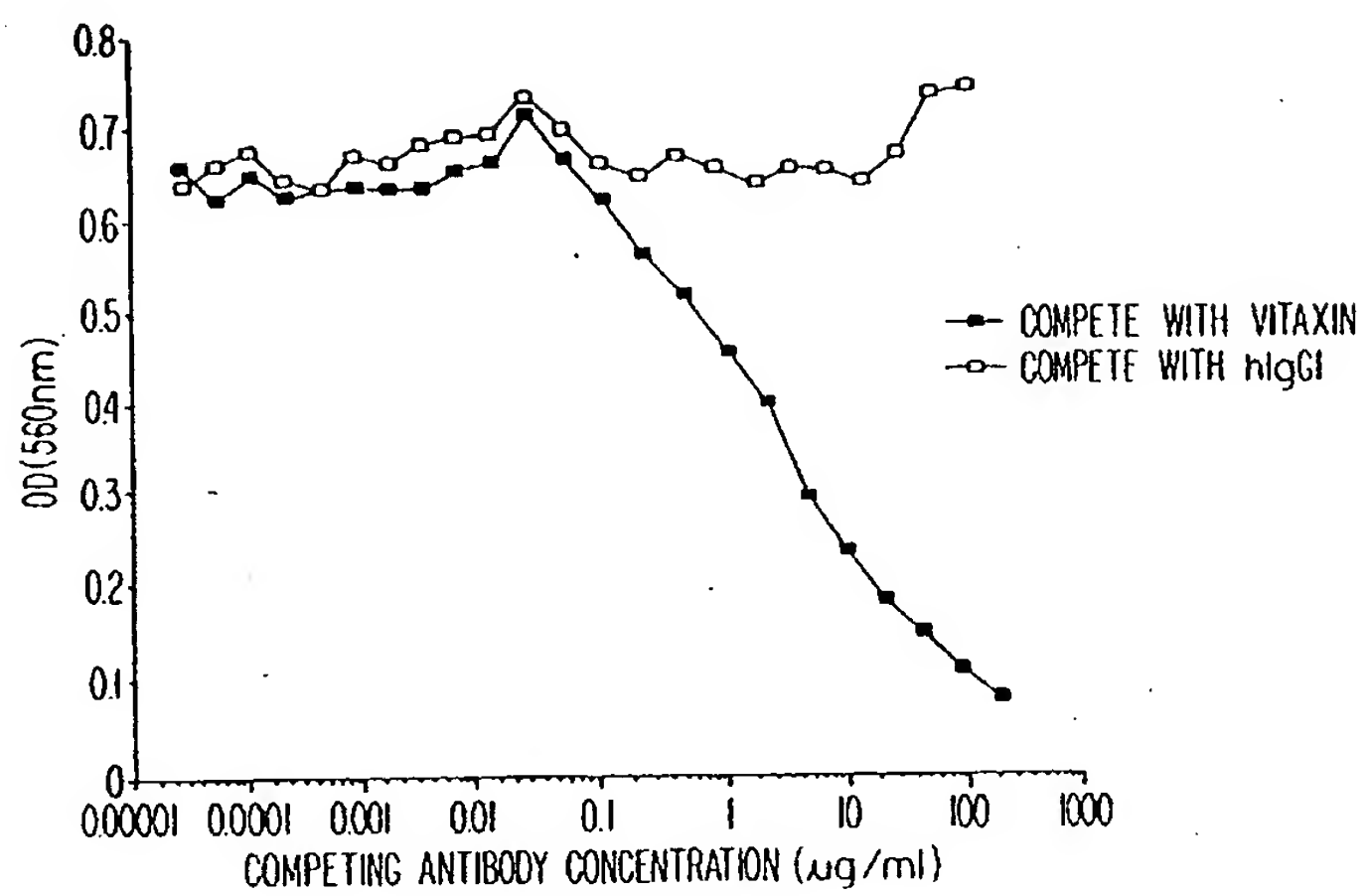


FIG. 4B

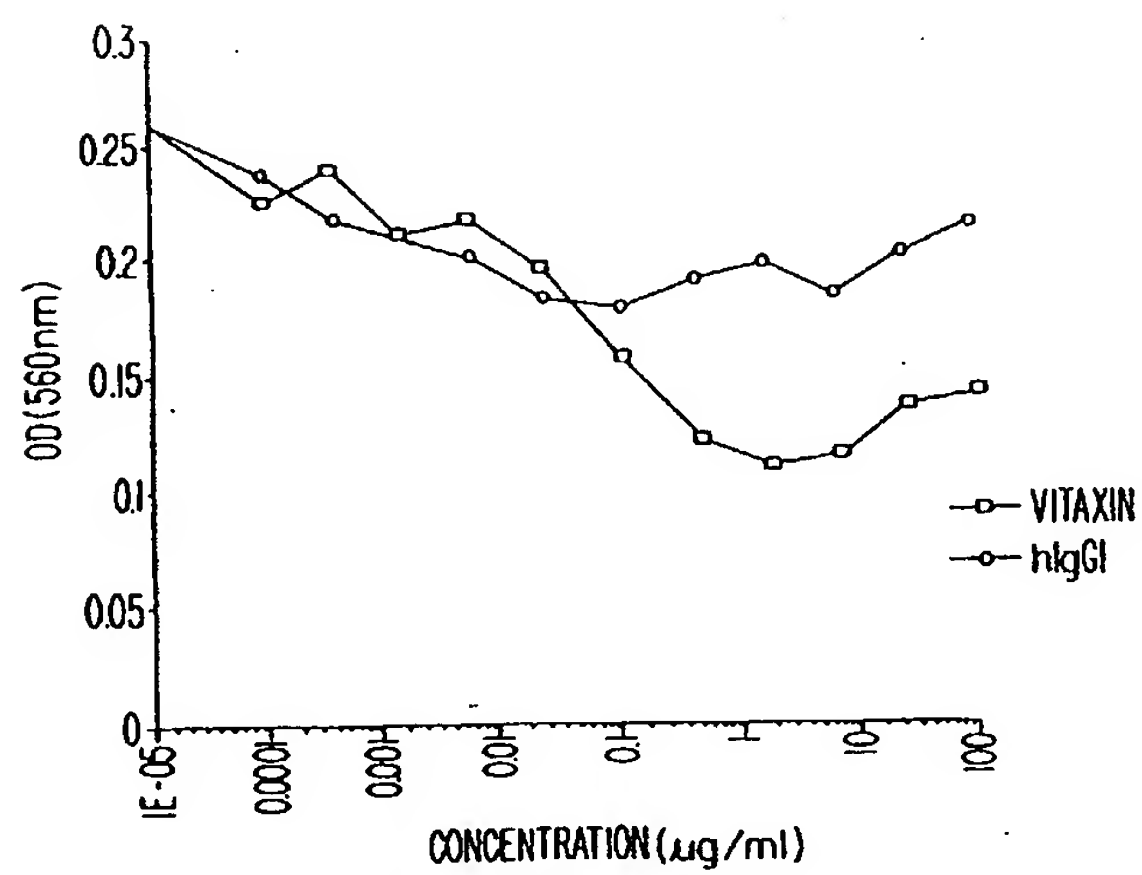


FIG. 4C

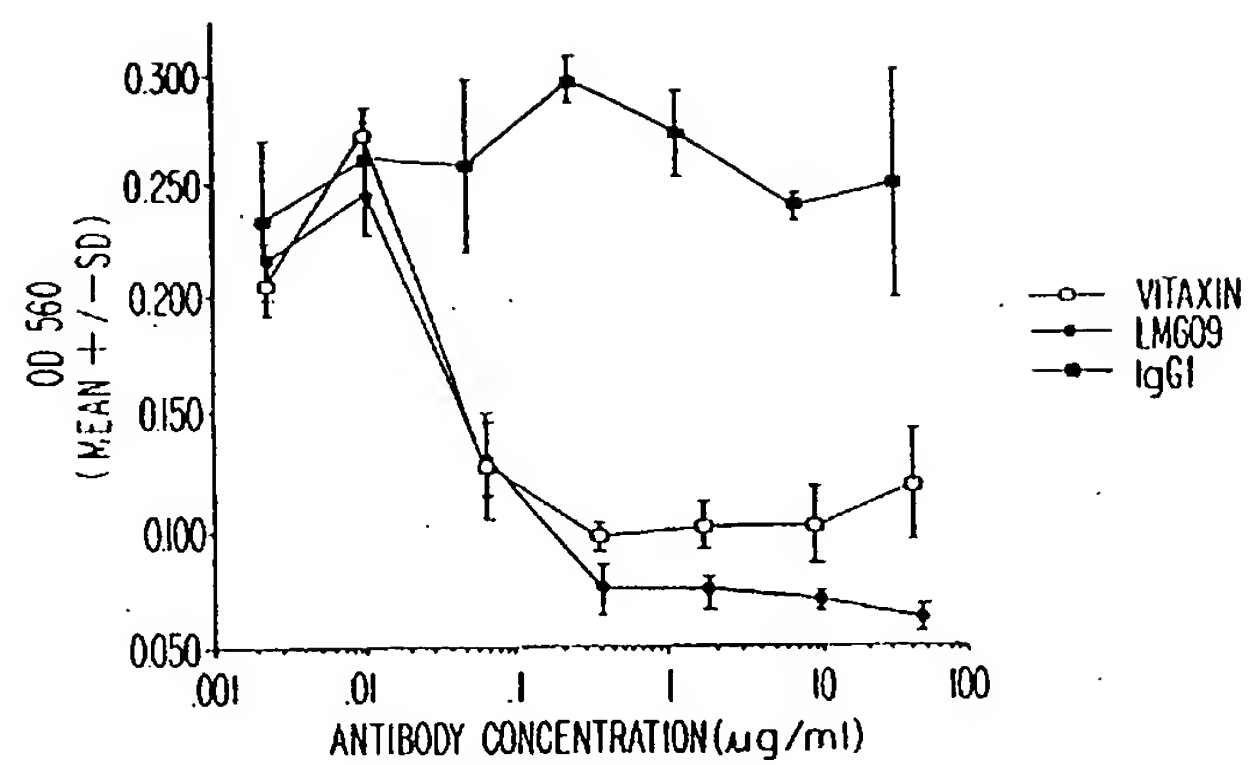


FIG. 5A

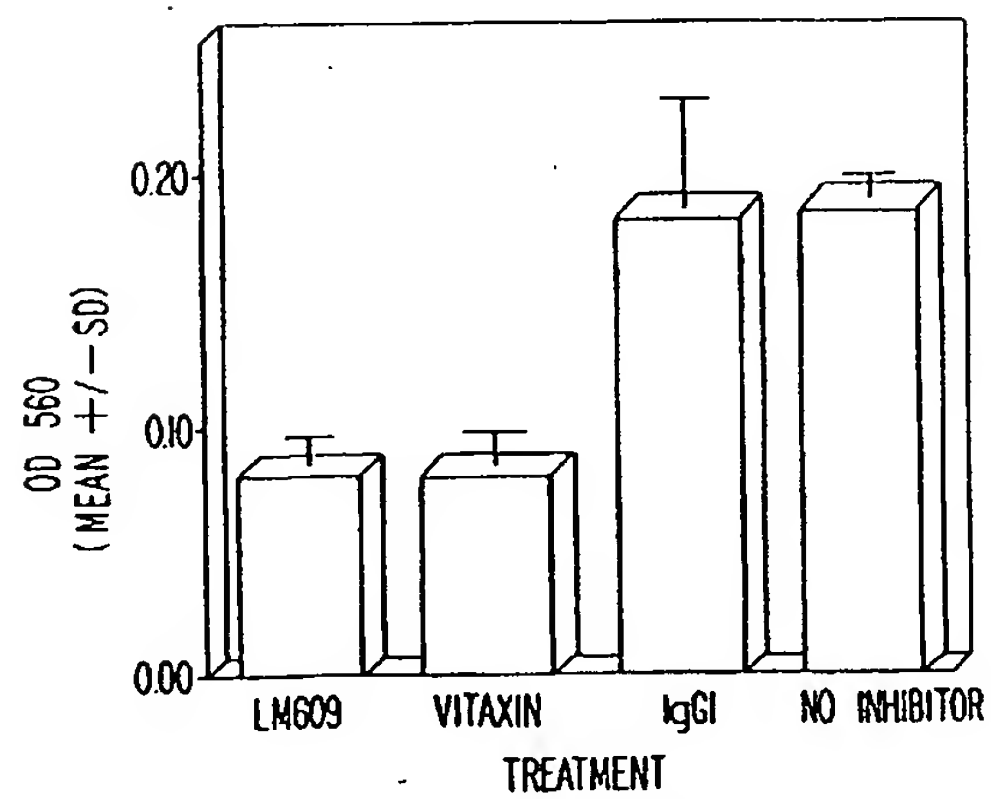


FIG. 5B

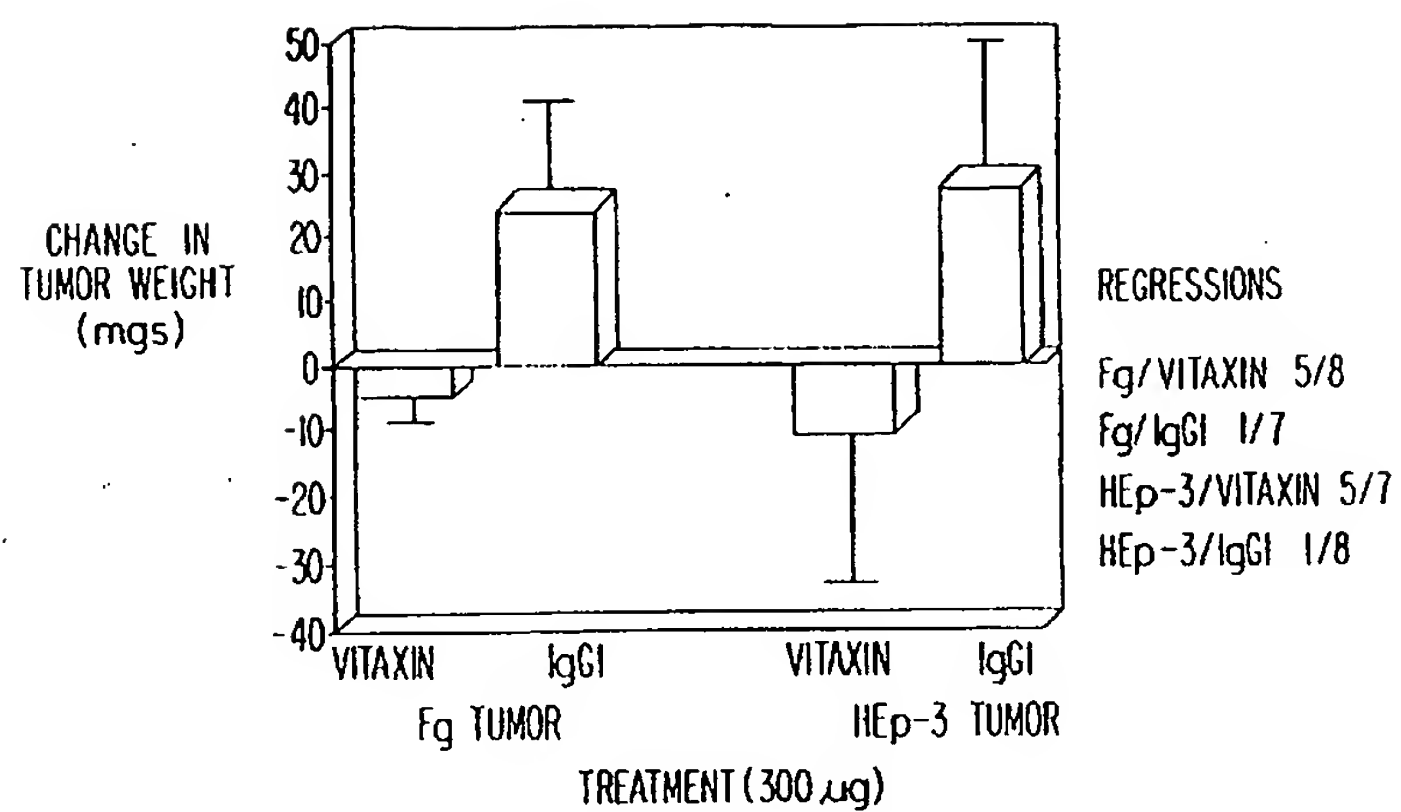


FIG. 6A

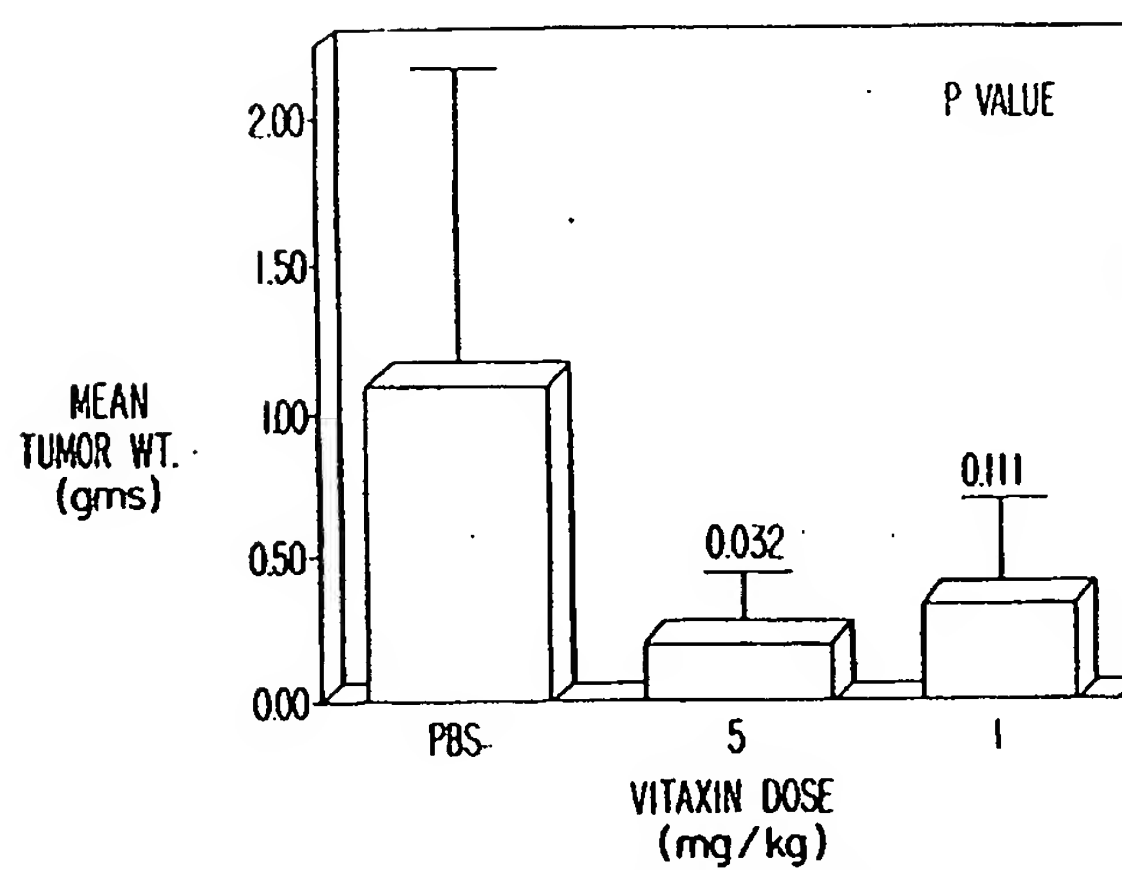


FIG. 6B

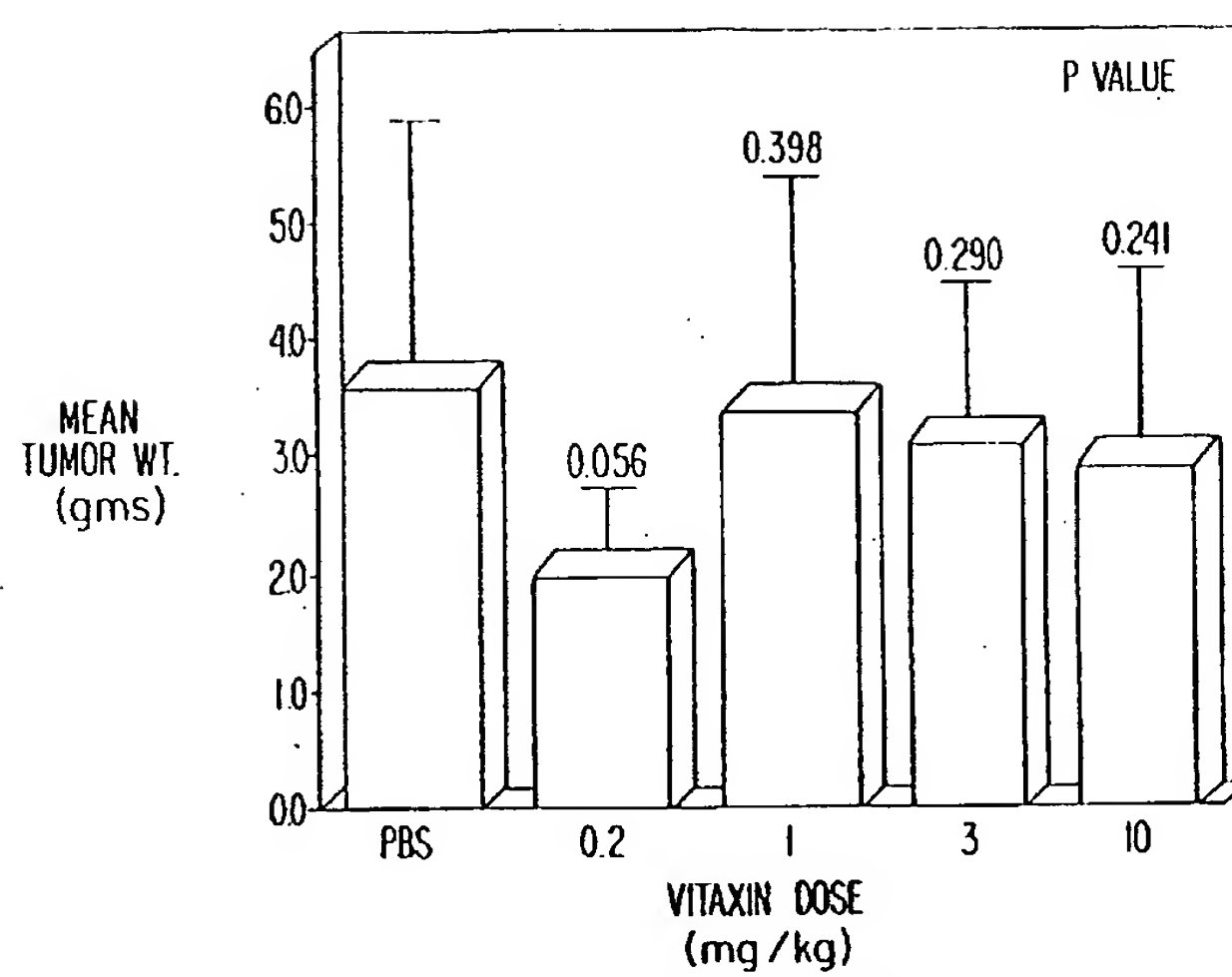


FIG. 6C

GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA Glu Ile Val Leu Thr 5 1	48
GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC Glu Arg Ala Thr 20 25	96
CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC Leu His Trp Tyr Gln Gln Arg Pro Gly Gln Ala Pro Arg 45 35	144
CGT/ATG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC Arg/Met Tyr Arg Ser Gln Ser 55 50	192
AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT Ser Gly Ser Gly Thr Asp 70 65	240
GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGT GGC AGC TGG CCT CAC Glu Asp Phe Ala Val 85 90	288
ACG TTC GGA GGG ACC AAG GTG GAA ATT AAG Thr Phe Gly Gly Thr Lys Val 105 100	321

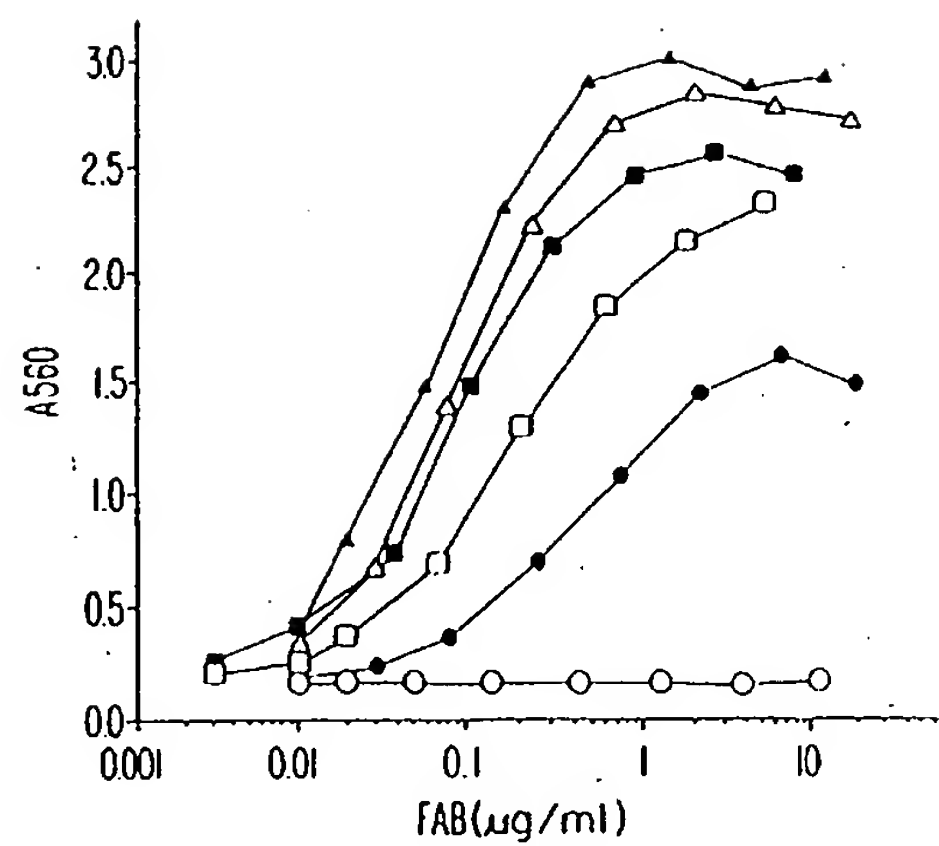


FIG. 8

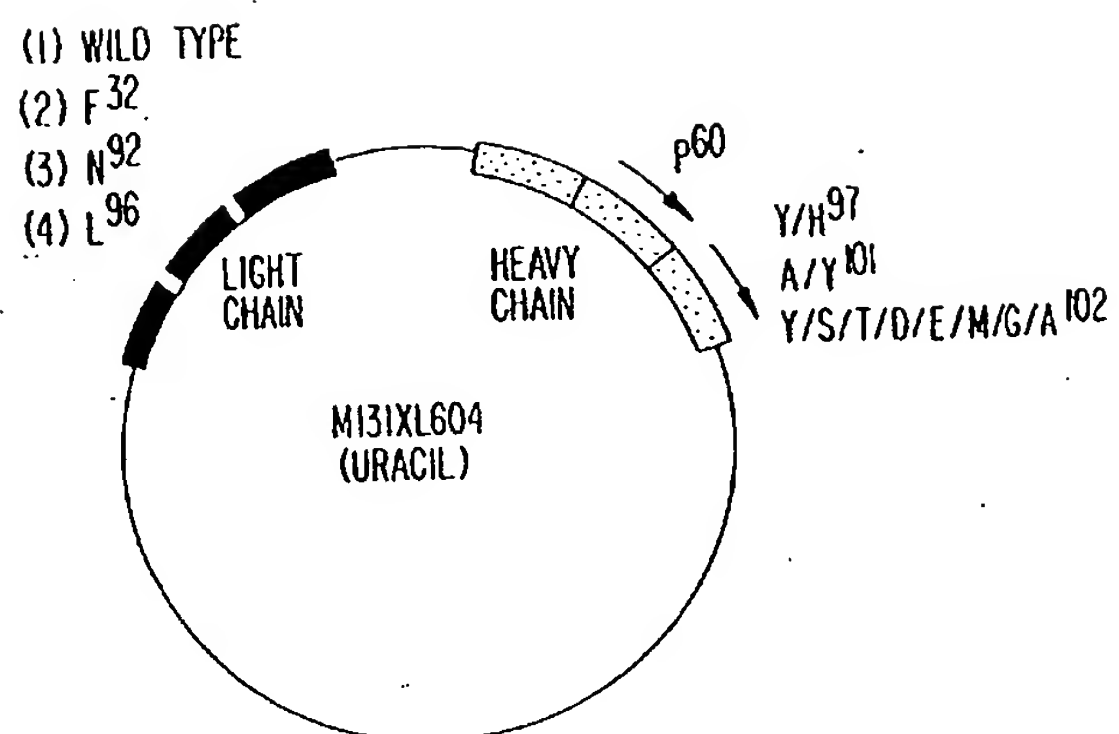


FIG.9

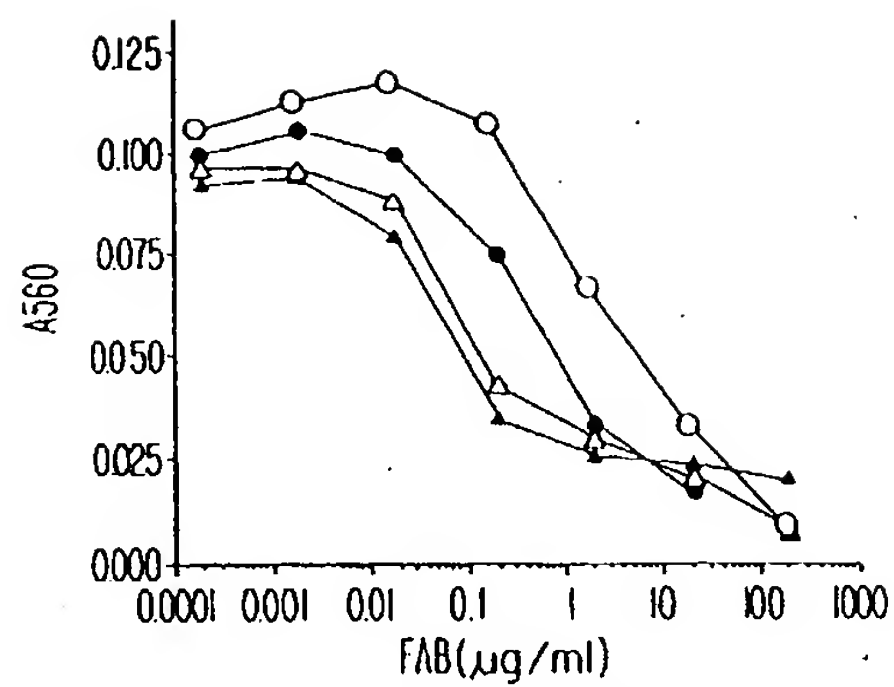


FIG. 10A

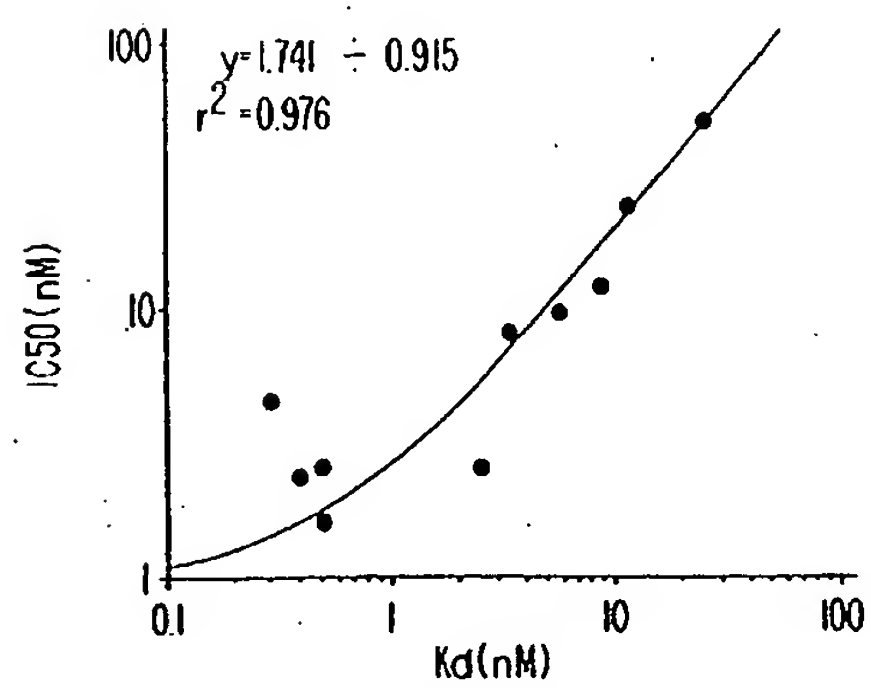


FIG. 10B

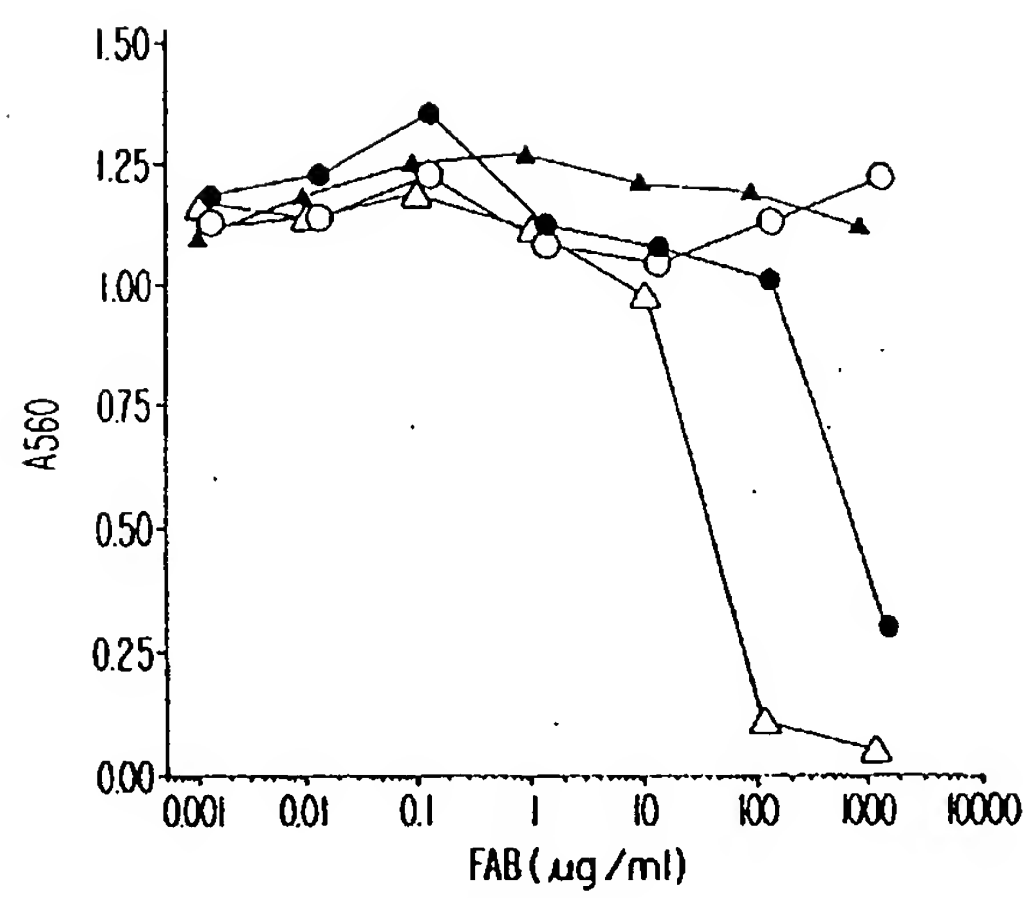


FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/17454

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/28 A61K39/395 C07K16/46 C12N15/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 33919 A (IXSYS INC) 6 August 1998 (1998-08-06) abstract page 1, line 8-12 page 5, line 8-12 page 8, line 27-30 page 22, line 1 -page 31, line 32 page 35, line 10 -page 39, line 3 page 39, line 17 -page 45, line 24 page 50; tables 4,5 page 51, line 29 -page 54, line 3 page 82, line 4 -page 100, line 24 --- -/--	1-33

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

10 November 2000

Date of mailing of the international search report

20. 11. 00

Name and mailing address of the ISA

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Authorized officer

Montrone, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/17454.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WU HERREN ET AL: "Stepwise in vitro affinity maturation of Vitaxin, an alphavbeta3-specific humanized mAb." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 95, no. 11, 26 May 1998 (1998-05-26), pages 6037-6042, XP000941358 May 26, 1998 ISSN: 0027-8424 . abstract page 6037, column 2, paragraph 2 -page 6038, column 1, paragraph 1</p>	1-33
Y	<p>DAVIES JULIAN ET AL: "Affinity improvement of single antibody VH domains: Residues in all three hypervariable regions affect antigen binding." IMMUNOTECHNOLOGY (AMSTERDAM), vol. 2, no. 3, 1996, pages 169-179, XP004070292 ISSN: 1380-2933 abstract</p>	1-33
Y	<p>CHOWDHURY P S ET AL: "Improving antibody affinity by mimicking somatic hypermutation in vitro." NATURE BIOTECHNOLOGY, (1999 JUN) 17 (6) 568-72. , XP000918985 abstract</p>	1-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/17454

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 21 to 24 are partially directed to a method of treatment of the human/animal body insofar as they refer to an in vivo method, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/17454

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9833919 A	06-08-1998	AU 6139198 A EP 0970217 A	25-08-1998 12-01-2000

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